PLASTICITY IN REPRODUCTIVE TRAITS

Meghan A. Guinnee

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CONTENTS

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Declaration

I declare that this thesis is entirely my own work, and has been written by myself, except where otherwise stated in the text and the collaborations outlined below. This work has not been submitted for any other degree.

This research was only possible due to collaboration with, and help from, many people, described below:

Chapter 5: A. Gardner did the mathematical modelling work and the majority of the writing and figures for section 5.3.

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ABSTRACT

Within the same species, different individuals have different life-history characteristics. Much of this is due to genetic differences – but much is due to 'phenotypic plasticity', defined by Pigliucci (2001) as "the property of a given genotype to produce different phenotypes in response to distinct environmental conditions." A 'maternal effect' is a specific type of phenotypic plasticity where an individual's characteristics are influenced by the environment or condition of its mother. Phenotypic plasticity and maternal effects can be adaptive, but need not be. In this thesis, I explore how an individual's environment, or the environment of its mother, affects its reproductive life-history characteristics (age at maturity, size at maturity, offspring size, offspring number). I attempt to explain observed responses using adaptive reasoning and/or mathematical modelling.

The trade-off between offspring size and number is one of the most studied areas of evolutionary biology, but we still do not have a complete understanding of why offspring (or egg) size variation exists within species. I explored a particular type of small clutch model, the life-history invariants proposed by Charnov and colleagues (Charnov & Downhower 1995; Charnov *et al.* 1995; Downhower & Charnov 1998), which make quantitative predictions regarding the relationship between clutch size and range in offspring (egg) size. I tested their predictions using the waterflea, *Daphnia magna* (freshwater crustacean), under closely-controlled, laboratory conditions. I found qualitative support for the model in that the range of mean egg sizes (averaged over clutch) decreased with increasing clutch size. However, this decrease was slower than predicted. I also tested the model using parasitic wasp data previously collected by other researchers. This allowed me to test the generality of the model over multiple species, and to examine whether the relationship holds for sexual species. Again, I found qualitative support for the model in that range in offspring size decreased with increasing clutch size. However, the decrease did not follow the quantitative predictions, and how well the data fit the model varied from

species to species. It is likely that genetic differences, along with the fact that the mothers produced both female and male offspring, added variability to the system.

I found that mean egg size decreases with increasing clutch size in *Daphnia*, and explored possible causes of this using a mathematical model. This pattern could be an adaptive response, if larger offspring have greater fitness advantages in food-limited environments. Alternatively, such a pattern can result from a minimum viable egg size. I also examined the fitness effects of hatching from a small or large egg in *Daphnia*. I found that offspring from food-limited mothers are larger, but that they mature later, and produce fewer, smaller offspring. This might be because offspring from food-limited mothers are programmed to be more cautious, investing resources into survival rather than reproduction.

I found that the nematode parasites *Strongyloides ratti* and *Nippostrongylus brasiliensis* mature at different rates depending on the efficacy of the host immune response, but that differences are species-dependent. In addition, female *N. brasiliensis* suffer decreased fecundity at higher densities, but only in hosts with fully-functioning immune systems. This suggests that the density-dependent effects often observed in parasitic nematodes are mediated by the host immune system.

This thesis reminds us that small differences in an individual's surroundings, or even its mother's surroundings, can profoundly affect when, how, and how successfully an animal reproduces. Often, these effects can be explained using adaptive reasoning, and/or mathematical modelling. When and how an animal reproduces is certain to have consequences for its fitness, and even the evolution of species. The implications of this, and possible future research directions are discussed.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Phenotypic plasticity

The aim of life-history theory is to explain the mind-boggling diversity of the life-history characteristics of organisms. That different species have vastly different life-history characteristics is obvious: the adult giant tortoise can live over 150 years, while the adult mayfly lives just one day. But there is also great diversity in life-history characteristics within species: a 200 year-old lodgepole pine at the foot of a mountain might be 100 feet tall, while another of the same age on the top of the mountain might only reach a few feet in height. The two trees are of the same species – how can such differences be explained?

Diversity can come about through genetic differences – within any species individuals will have different genetic backgrounds which will undoubtedly influence their life-history traits. But an organism's characteristics are not solely dependent on its genetic inheritance: its environment also plays a role. For example, the seedling that finds itself, by chance, under the shade of a large tree will grow slower and reproduce later than its sibling that finds itself in full sunlight. These differences are an example of 'phenotypic plasticity', defined by Pigliucci (2001) as "the property of a given genotype to produce different phenotypes in response to distinct environmental conditions."

The above example is the simplest type of phenotypic plasticity: the tree grows slower because it does not receive a lot of sunlight. But phenotypic plasticity can also be adaptive: some organisms have evolved the ability to sense their environment and alter their characteristics to maximise fitness, and organisms that live in highly variable environments are often able to use environmental cues to alter their life-history strategies (Moran 1992; Scheiner 1993; Pigliucci 2001). For example, many amphibians in temporary ponds can adjust speed of metamorphosis in line with water levels – the less water, the faster they develop (Denver 1997; Denver *et al.* 1998). This response is reversible if water levels increase (Denver *et al.* 1998), and likely mediated by stress hormones (Denver 1997; Denver 1999). Likewise, there is evidence that insects use population density cues to assess the risk of becoming infected with pathogens, and adjust allocation of resources to disease resistance accordingly (Wilson & Reeson 1998; Wilson *et al.* 2001; Wilson *et al.* 2002). *Daphnia* adjust their age and size at maturity in the presence of a predator: exposing a *Daphnia* to chemicals from predators that prey on small *Daphnia* causes it to mature later and larger, while exposing it to chemicals from predators that prey on large *Daphnia* result in earlier maturation at a smaller size (Stibor 1992; Weider & Pijanowska 1993; Stibor & Luning 1994).

A 'maternal effect' is a common type of phenotypic plasticity where an individual's characteristics are influenced by the environment or condition of its mother. For example, mothers experiencing poor or changing environmental conditions produce a greater proportion of diapausing offspring (or offspring producing diapausing eggs) in a variety of plants and animals (Roach & Wulff 1987; Mousseau & Dingle 1991; Fox & Mousseau 1998; Mousseau & Fox 1998; LaMontagne & McCauley 2001; Alekseev & Lampert 2001). Likewise, radish seeds produced by plants grazed by caterpillars contain more defensive chemical compounds, and the plants that grow from these seeds are less nutritious to caterpillars than plants derived from ungrazed plants (Agrawal *et al.* 1999). Mothers can influence the fitness of their offspring through resource provisioning, and offspring that are larger or emerge from larger propagules are generally more fit (e.g. McGinley *et al.* 1987; Roff 1992; McEdward

1996; Fox & Mousseau 1998; Mousseau & Fox 1998; Einum & Fleming 2000). Optimal propagule size can depend on environment, and it has been suggested that it might be in a mother's best interest to provision resources to her offspring differently under different environmental conditions (Hutchinson 1951; Green 1966; Berven & Chadra 1988; Fleming & Gross 1990; Gliwicz & Guisande 1992; Westoby *et al.* 1992; Bernardo 1996b; Rees & Westoby 1997). Like other types of phenotypic plasticity, a maternal effect can be, but need not necessarily be, adaptive (Bernardo 1996a). Although they can greatly impact the fitness and life-history of individuals, and could affect selection (Bernardo 1996a; Mousseau & Fox 1998), many aspects of maternal effects are just now beginning to attract the attention of researchers.

In this thesis, I explore how an individual's environment, or the environment of its mother, affects its reproductive life-history characteristics (age at maturity, size at maturity, offspring size, offspring number). I attempt to explain observed responses using adaptive reasoning and/or mathematical modelling. I use *Daphnia* and parasitoid wasps to test mathematical models predicting the relationship between egg size and clutch size. I also explore the fitness consequences of hatching from a small or large egg in *Daphnia*. Using parasitic nematodes, I examine whether parasites can sense the efficacy of their host's immune system and alter their age at maturation to maximise fitness.

In the remainder of this General Introduction, I discuss the model systems I use, and the aims of this thesis. In the next chapter, I give an overview of optimal clutch size theory, explain generally what an invariant is, and discuss specifically the life-history invariants I explore in this thesis. Specifics relevant to each chapter can be found in the introduction of those chapters.

1.2 Experimental organisms

Daphnia magna

Daphnia, or water fleas, are small, freshwater crustacea found in ponds throughout the temperate regions of the world. *Daphnia* generally reproduce clonally, but can also reproduce sexually when exposed to environmental stressors. Sexual reproduction leads to the production of ephippia, or diapausing eggs, which can survive harsh environments where survival is otherwise unlikely. For the experiments described in this thesis, *Daphnia* were kept under conditions where only clonal reproduction occurs.

Daphnia are an ideal study organism for many reasons: (1) much is known about their life-history, physiology, population dynamics, genetics and evolution (Chapters 3, 4 and 5; Green 1956; Hebert 1978; Peters and de Bernardi 1987; Colbourne & Hebert 1996); (2) the eggs are visible through the carapace, allowing us to tell when a *Daphnia* is mature and easily count how many eggs she has produced; (3) we can control the number of eggs produced by a single *Daphnia* by manipulating food availability; (4) we can experimentally manipulate, with extreme precision, the amount of resources a female has by varying the amount of available food; (5) as they are clonal, we can compare genetically identical individuals; and (6) *Daphnia* have a short generation time, and are easily kept in culture, allowing for quick experiments and large sample sizes. Throughout this thesis, I use a clone of *Daphnia magna* that originated in the Gaarzerfeld pond, Northern Germany. I chose this clone as much is known about it, as *Daphnia* from this pond have been the focus of numerous studies of parasitism and life-history evolution (Little & Ebert 2001; Little *et al.* 2002).

Daphnia exhibit phenotypic plasticity in a number of traits, including: offspring number (Spitze 1992; Enserink *et al.* 1993; Burns 1995), offspring (or egg) size (e.g. Lynch 1989; Tessier & Consolatti 1991; Ebert 1993), resting egg production

(Slusarczyk 1995; Pijanowska & Stolpe 1996), morphology (Harvell 1990; Spitze 1992; Tollrian 1995), age at maturity (Spitze 1992; Weider & Pijanowska 1993; Giebelhausen & Lampert 2001), growth (Burns 1995), and body size (Weider & Pijanowska 1993; Stibor & Luning 1994; Tollrian 1995), to name but a few. Likewise, maternal environment can influence many characteristics in *Daphnia* offspring, including: resting egg production (LaMontagne & McCauley 2001; Alekseev & Lampert 2001), susceptibility to parasitism (Little *et al.* 2003), survival (Lynch & Ennis 1983; Gliwicz & Guisande 1992; Cleuvers *et al.* 1997), morphology (Agrawal *et al.* 1999), growth (Lynch & Ennis 1983), and reproduction (Lynch & Ennis 1983).

In Chapter 3, I test Charnov and colleague's invariant rules (see Chapter 2 for discussion; Charnov *et al.* 1995; Charnov & Downhower 1995; Downhower & Charnov 1998) using *Daphnia.* This is the most in-depth test of these invariants to date. In Chapter 5, I explore the effects of a minimum egg size and selection for smaller eggs at higher resource availability on mean egg size and range in egg size using a mathematical model. In Chapter 6, I examine whether differences in egg size in *Daphnia* (as described in Chapter 3) translate into fitness differences for the *Daphnia* hatching from those eggs.

Parasitoid wasps

The larvae of parasitoid wasps feed exclusively on the body of their arthropod host, eventually killing it. Depending on the species, one or more larvae can develop on the same host. Recently, parasitoid wasps have received attention due to their successful use as biocontrol of agricultural pests (Godfray 1994), and parasitoids have been used to test numerous aspects of evolutionary biology, including the evolution of: clutch size (Godfray 1987; Godfray & Parker 1991; Hardy *et al.* 1992; Petersen & Hardy 1996; Mayhew & Hardy 1998), body size (van den Assem *et al.* 1989; Petersen & Hardy 1996), siblicide behaviour (Mayhew & Hardy 1998), and

sex ratio (Green *et al.* 1982; Hardy & Mayhew 1998; Hardy *et al.* 1998; West *et al.* 2001), to name but a few (also reviewed by Godfray 1994).

Parasitoid wasps are an ideal model for studying resource sharing in small clutches as: (1) many species produce small, but variable, sized clutches (Godfray 1994; Mayhew & Hardy 1998); (2) datasets on many species are available from parasitoid researchers, making it easy to carry out analyses on multiple species; and (3) we can measure clutch resources by measuring host size, as the host provides all the resources for wasp offspring until they mature to adulthood. In Chapter 4, I test the generality of Charnov and colleague's invariant rules (Charnov *et al.* 1995; Charnov & Downhower 1995; Downhower & Charnov 1998) using multiple species of parasitoid wasps.

Parasitic nematodes

Strongyloides ratti and *Nippostrongylus brasiliensis* are gastrointestinal nematode parasites of rats. Adult parasites of both species inhabit the mucosa of the small intestine. In the intestine, *S. ratti* are exclusively parthenogenic females (Viney 1994), while *N. brasiliensis* reproduce sexually. Eggs of both species are shed into the intestine and pass with host faeces to the external environment where further development takes place resulting in infective third stage larvae (iL3s). When an iL3 comes into contact with a rat, it burrows through host skin and migrates to the intestine. *S. ratti* larvae can alternatively develop into free-living adults, which reproduce sexually. The sexually-produced offspring then develop into iL3s which are infective to rats (Anderson 1992).

S. ratti and *N. brasiliensis* are common laboratory model systems: much is known about their lifecycle, genetics, host specificity, reproduction, and the effect of the host immune response (Twohy 1956; Ogilvie & Jones 1971; Moqbel & McLaren 1980; Kassai 1982; Anderson 1992; Viney 1994; Gemmill & West 1998; Gemmill *et*

al. 2000; Paterson & Viney 2002; Wilkes *et al.* 2004). *S. ratti* exhibits phenotypic plasticity in that the immune status of the host influences whether larvae develop into sexual adults (Gemmill *et al.* 1997). This suggests that the worms are able to sense the host immune environment and adjust life-history accordingly.

In Chapter 6, I test whether *S. ratti* and *N. brasiliensis* are able to sense the efficacy of the host immune system and adjust their age at maturation in line with nematode mortality rate. In Chapter 7, I use *N. brasiliensis* to explore the effects of nematode density on nematode size and fecundity. I also examine how this relationship is affected by host immune status.

1.3 Thesis aims

In this thesis, I carry out a number of experiments to explore how an individual's environment, or the environment of its mother, affects reproductive life-history traits. The specific questions this thesis examines are:

1) Does variation in offspring (or egg) size follow Charnov and colleague's invariant rules (Charnov & Downhower 1995; Charnov *et al.* 1995; Downhower & Charnov 1998; Chapters 2, 3 and 4), and how general are the rules across species (Chapter 4)?
2) How do a minimum viable egg size, and selection for smaller eggs at higher resource levels, affect mean egg size and Charnov *et al.*'s invariant rule (using mathematical modelling; Chapter 5)?
3) What are the fitness implications of hatching from a small or large egg, or being a small or large offspring (Chapter 6)?
4) Can parasites sense the immune status of their host and adjust their age at maturity accordingly (Chapter 7)?
5) Do observed density effects in parasitic nematodes result from increased host immune response or increased competition between worms at high nematode densities (Chapter 8)?

CHAPTER 2

THE TRADE-OFF BETWEEN OFFSPRING SIZE AND OFFSPRING NUMBER, AND WHAT IT HAS TO DO WITH INVARIANTS

2.1 Summary

While the trade-off between offspring size and offspring number is one of the most studied areas of evolutionary biology, we still do not have a complete understanding of why variation in offspring size exists within species. Recently, small clutch models have made invariant predictions about how the range in offspring size should decrease with increasing clutch size. I test these models in Chapters 3 and 4 of this thesis. Here, I give the background information in invariants and clutch size modelling necessary to the understanding of those chapters.

2.2 Life-history invariants

Life-history invariants are unchanging relationships between two life-history traits with the same units; these relationships often remain unchanged over a variety of species. For example, in mammals, yearly litter size (in daughters) scales with age at maturity to a slope of -1 (figure 2.1; Charnov 1993). While it is often difficult to make quantitative life-history predictions without an acute understanding of the biology of an organism, invariants allow us to examine the life-history traits of multiple individuals, populations or species, without the need of a lot of background

information. Invariants have been described for such diverse topics as sex allocation, age at maturity, and cellular maintenance rates, to name but a few, and in animals as varied as mammals, fish, invertebrates and reptiles (Charnov 1993; Charnov & Skuladottir 2000; Charnov 2001; Allsop & West 2003a; Allsop & West 2003b).

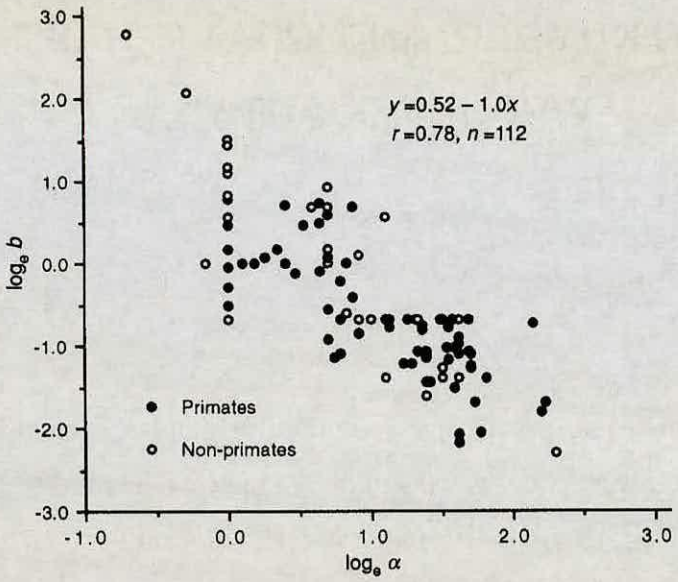

Figure 2.1: Log of yearly litter size (*b*) versus log of age at maturity (α) in mammals (slope = -1). Data are from Hennemann 1983 and Ross 1992. Figure is reproduced directly from Charnov (1993).

Invariants can be seen as 'universal laws of evolution' which describe the fundamental relationships underpinning life-history evolution. However, their usefulness can be called into question as it is often difficult to understand the underlying mechanisms generating the invariant, or whether those underlying mechanisms are biologically interesting. In the above example (figure 2.1), it might be that those mammals that mature later put more effort into offspring care than those that mature earlier. Or, those mammals that put less effort into reproduction might have a greater survival rate and therefore may have evolved to mature later. Alternatively, the relationship might be the result of some physiological constraint. As to the usefulness of invariants, Charnov, one of the main proponents of invariant

research, states, 'We can search for invariant outcomes in the relative structure of life histories, and *then use evolutionary life history theory to probe for the underlying symmetries which we guess to be causal*' (Charnov 1993). Essentially, invariants can point out areas of research that may reveal the fundamental pressures that shape evolution.

For example, Gemmill *et al.* (1999) demonstrated a relationship between mortality and age at maturation in parasitic nematodes that explains approximately half the variation in maturation time across a variety of species. Gemmill *et al.* (1999) suggests that this relationship arises from the fact that larger worms are more fecund, but also take longer to reach maturity, increasing the risk of death before reproduction. Therefore there exists a trade-off between fecundity per unit time and size (age) at reproduction – where mortality is high, worms that mature quicker (and are smaller, with lower fecundity per unit time) are favoured. Where mortality is low, worms that take longer to mature (and are larger, with higher fecundity per unit time) are favoured. Such a hypothesis could be tested in the laboratory by altering mortality regimes and observing how the organisms respond.

Similarly, Allsop and West (2003a,b) found that, for organisms that change sex, the sex change occurs at the same relative body size (when compared to the maximum body size). Specifically, sex change occurs at about 72% of maximum body size, regardless of the details of the sex change or the life-history characteristics of the organism. Multiple taxa were found to follow this invariant, from a 2mm shrimp to a 1.5m fish. Some start as males and change to females, some change from females to males, some have harems, some exhibit male 'sneaking' behaviour. The underlying mechanisms driving the invariant are unknown. Perhaps the relationship arises as a result of the trade-off between size and age at maturation. Alternatively, the cause might be an as-yet-undiscovered physiological constraint. Such hypotheses are amenable to testing through further research, and the underlying causes of this invariant can be explored mathematically to further understand the possible underlying mechanisms (Gardner *et al. In Press*).

In Chapters 3 and 4, I use *Daphnia* and parasitoid wasps to better understand a proposed life-history invariant with the aim of explaining variation in offspring size in small clutches. In Chapter 5, I further expand upon this invariant to better understand the evolutionary causes of differences in offspring size at different resource levels and different clutch sizes. Below, I expand upon the reasoning behind this small clutch invariant.

2.3 Modelling optimal clutch size: successes

A well-studied problem in life-history theory is the trade-off between offspring size and offspring number (Roff 1992; Stearns 1992). Evolutionary models exploring optimal clutch size have typically used the approach described by Smith and Fretwell (1974) which I will briefly outline below. The model assumes that: (1) the amount of resources (R) for each clutch is fixed; (2) survival to adulthood (S) depends on the amount of resources an offspring receives (I); and (3) resources are divided equally between members of a clutch. A mother with R resources for a single clutch can produce R/I number of offspring in that clutch. Each offspring has a survival probability of $S(I)$. Therefore, the number of offspring which survive to adulthood (or the clutch fitness, F) from a single clutch is given by: $(R/I)*(S(I))$. As the resources for an individual offspring increases, the likelihood of that offspring surviving to adulthood also increases. But, for any given R, the more resources each offspring receives, the fewer offspring a mother can produce. Offspring (egg) size is used as a measure of resources, as offspring (egg) size is correlated with offspring fitness (as discussed in Chapter 1). There will exist an optimum offspring (egg) size (I^*) which maximises clutch fitness (F) for any R. I^* is the point where the line through the origin is tangent to the function relating size to offspring fitness (figure 2.2).

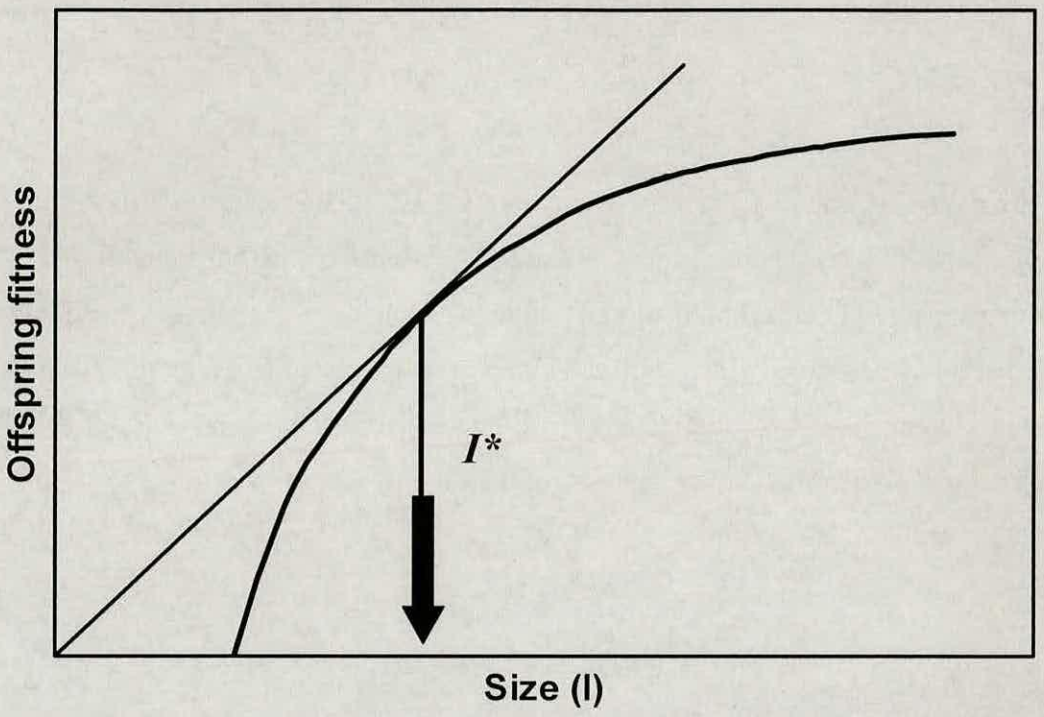

Figure 2.2: The curve relating offspring size to offspring fitness. Offspring fitness increases with parental investment per offspring (*l*), here measured as offspring size. In theory, the offspring size-offspring fitness curve could take on any shape. The optimal offspring size (*l**) that maximises maternal fitness is defined as offspring size where the line through the origin is tangent to the curve.

2.4 Modelling optimal clutch size: problems ... and solutions

The above method has proven highly useful (Roff 1992). However, clutch size is an integer trait, and the above model treats it as continuous. This can lead to problems at small clutch sizes (Smith & Fretwell 1974; Ebert 1994; Charnov & Downhower 1995; Charnov *et al.* 1995). For example, if resources exist intermediate to that required for producing a clutch of size one or two, a mother then has to decide between producing one offspring that will be larger-than-optimum size or two offspring that will be smaller-than-optimum size. This decision is non-trivial, as the one offspring would then be twice the size of each of the two offspring; this will presumably have large fitness effects for those offspring. A mother should switch from producing a clutch of size of 1 to a clutch size of 2 when the clutch fitness (F)

associated with one offspring of size I is equal to the F of two offspring each of size $I/2$.

Charnov and colleagues (Charnov & Downhower 1995; Charnov *et al.* 1995) realised that this causes the range of possible offspring sizes within a clutch size to decrease in a predictable and invariant manner as clutch size increases. Specifically, that variance in offspring size (between clutches) is predicted to decrease with increasing clutch size, whereby the ratio of the range of offspring sizes for clutches of size i and j offspring is the reciprocal of the ratio of clutch sizes (Charnov *et al.* 1995; Charnov & Downhower 1995; West *et al.* 2001). This is given formally by:

$$\frac{I_{\max\_i} - I_{\min\_i}}{I_{\max\_j} - I_{\min\_j}} = \frac{C_j}{C_i} \qquad (2.1)$$

where I is offspring size, C is clutch size, $I_{max\,i}$ and $I_{min\,i}$ are the maximum and minimum size of offspring in clutches of C_i young, i and j are broods of different sizes, and $i=j+1$. Figure 2.3 graphically depicts this relationship, which I refer to as Charnov *et al.*'s invariant rule. This invariant has been shown to hold for a variety of resource investment/offspring survival trade-off functions (Charnov *et al.* 1995). This invariant has been tested in a fish (*Gambusia hubbsi*; Charnov & Downhower 1995), and two species of parasitoid wasps (*Laelius pedatus*, Mayhew 1998; *Achrysocharoides zwoelferi*, West *et al.* 2001). In general, these studies found that range in offspring (or egg) size decreased with increasing clutch size, but not necessarily at the rate predicted by the model.

This model makes several assumptions: (1) Optimal offspring size (I^*) is constant, regardless of clutch size; (2) if offspring share resources before emerging (e.g. those within the same womb or developing on or in the same host), competition between offspring is constant over clutch size; (3) mothers can accurately assess the amount of resources available to their clutch; and (4) mothers do not employ a 'bet-hedging'

strategy whereby they provision offspring with greater resources than might otherwise be expected.

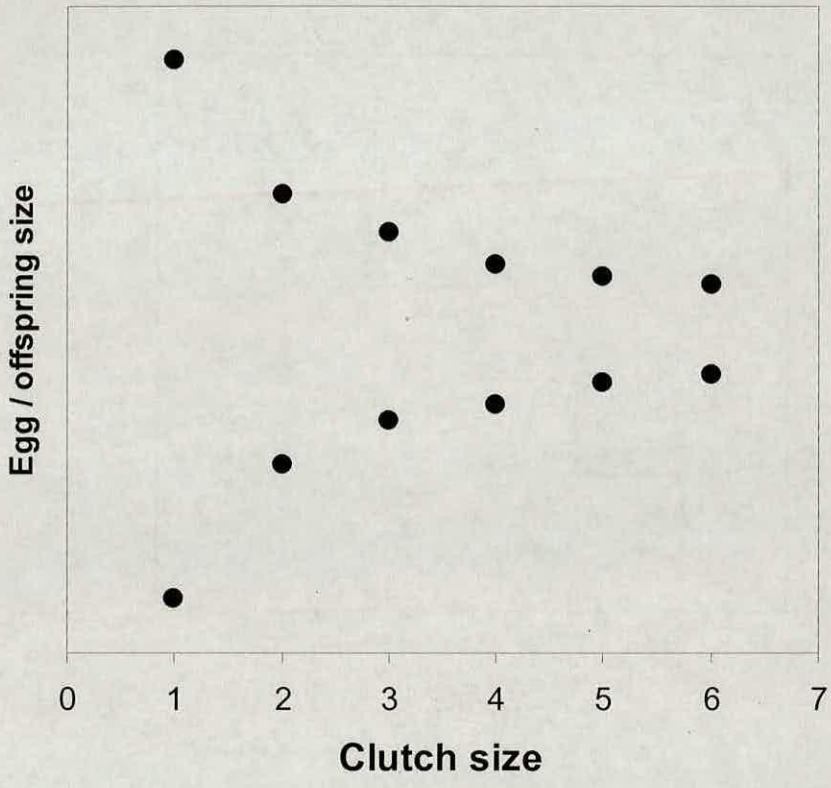

Figure 2.3: The predicted relationship between range in offspring (or egg) size and clutch size (as described by Equation 2.1). The upper point refers to the largest offspring size and the lower point to the smallest offspring size predicted for that clutch size. The distance between the points represents the total range of offspring sizes expected for that clutch size.

Downhower and Charnov (1998) and Mayhew (1998) also predict that the range of total resources devoted to reproduction (R) for any given clutch size should be constant over clutch size. In other words, the amount of extra resources necessary to go from a clutch size of C to one of C+1 should be constant regardless of C. This leads to the prediction that the relationship between clutch size (C) and resources (R) should be linear (figure 2.4). I refer to this as the Downhower and Charnov's invariant rule (1998). Downhower and Charnov (1998) found support for this

invariant rule in a fish (*Gambusia hubbsi*), while Mayhew (1998) found that, in a parasitoid wasp (*Laelius pedatus*), variance in host weights increased with increasing clutch size, suggesting that offspring were 'costlier' in larger clutches.

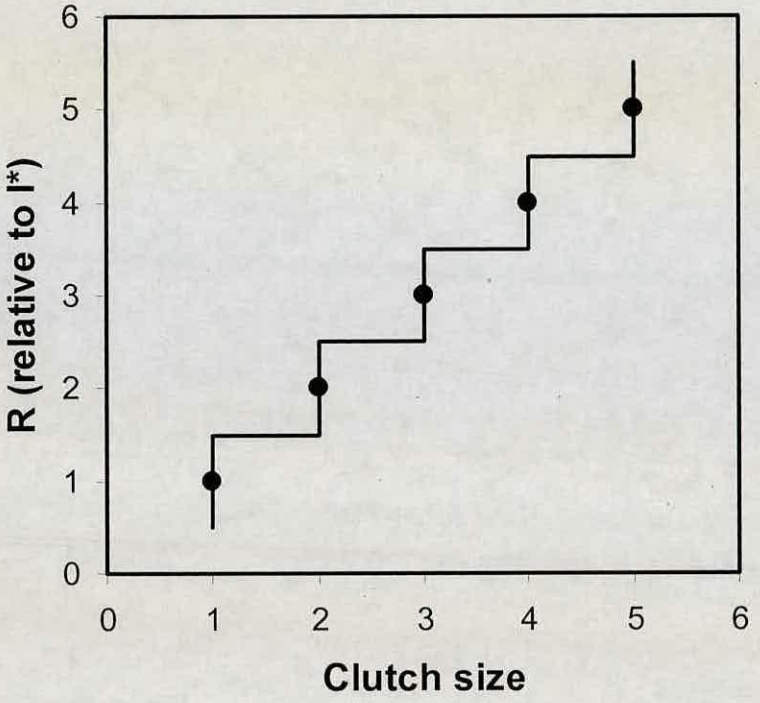

Figure 2.4: The predicted relationship between clutch size and resources for reproduction (*R*) relative to the optimum offspring size (*l**). The line represents all predicted possible clutch size/reproductive effort combinations. The black dots represent the average reproductive effort for each clutch size.

Invariant rules are independent of underlying trade-offs, such as the relationship between offspring size and fitness, meaning that these invariants in theory should hold for any organism that produces clutch sizes within the necessary range (1-6). If the invariants are found not to hold for certain organisms, this can suggest the assumpti

ons of clutch size models do not hold, and indicate areas for future research. To date, there have been few tests of these invariant predictions (but see Charnov *et al.* 1995; Mayhew 1998; Downhower & Charnov 1998; West *et al.* 2001), and there has yet to

be a comprehensive, experimental test of these invariant predictions. All previous tests have been carried out on sexually reproducing species, meaning that variation due to genetic and environmental factors is impossible to disassociate. Other tests were carried out in the field (Charnov *et al.* 1995; Downhower & Charnov 1998), making it impossible to control for environment. I test the above invariant rules using genetically identical *Daphnia* in the laboratory (Chapter 3). I also test the generality of the invariant rules using multiple species of parasitoid wasps (Chapter 4).

CHAPTER 3

TESTING SMALL CLUTCH SIZE MODELS USING *DAPHNIA*

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3.1 Abstract

Life-history theory predicts that, for small clutches, variance in egg size (between clutches) should decrease in a predictable invariant manner as clutch size increases. To test this, we studied *Daphnia magna* at 350 different food treatments and recorded the number of eggs and the volume of each egg for their first clutch. As predicted, we found that: the relationship between clutch size and resources devoted to reproduction was linear, variance in egg volume decreased with increasing clutch size, and resources were shared relatively equally between the eggs in a clutch. However, we found that the rate at which the range of egg volumes decreased with clutch size was slower then predicted. We discuss possible explanations for this discrepancy, including a lower limit on the volume of eggs that are produced and selection for smaller eggs when food is abundant. Consistent with these, we found that mean egg volume decreased with increasing clutch size.

3.2 Introduction

Evolutionary models exploring optimal clutch (litter) size have typically used the approach described by Smith and Fretwell (1974), which models the trade-off between offspring size and offspring number assuming: (1) that mothers have a finite amount of resources available for each clutch, and (2) better provisioned offspring are fitter. However, as the predicted optimal clutch size is often not an integer value, these models must round to the nearest clutch size. While this approach generally makes useful approximations at larger clutch sizes, it breaks down for smaller clutch sizes (Smith & Fretwell 1974; Ebert 1994; Charnov & Downhower 1995). For example, if a mother has resources intermediate to that required for producing a clutch size of one or two, she then has to decide between producing one larger-than-optimum sized offspring or two smaller-than-optimum sized offspring. Recently, a number of theoretical models have addressed this problem and explicitly examined the consequences of small integer numbers for clutch size evolution (Ebert 1994; Charnov & Downhower 1995; Charnov *et al.* 1995; Downhower & Charnov 1998; West *et al.* 2001).

A novel and useful feature of these small integer models is that they make quantitative predictions that do not depend upon difficult to measure life-history details and underlying trade-offs. Specifically, Charnov, Downhower and colleagues have developed two life-history invariant rules. First, variance in offspring size (between clutches) is predicted to decrease with increasing clutch size, whereby the ratio of the range of offspring sizes for clutches of size i and j offspring is the reciprocal of the ratio of clutch sizes (Charnov *et al.* 1995; Charnov & Downhower 1995; West *et al.* 2001). This is given formally by:

$$\frac{I_{\max\_i} - I_{\min\_i}}{I_{\max\_j} - I_{\min\_j}} = \frac{C_j}{C_i} \qquad (3.1)$$

where i is offspring size, C is clutch size, $I_{max\,i}$ and $I_{min\,i}$ are the maximum and minimum size of offspring in clutches of C_i young, i and j are clutches of different sizes, and $i=j+1$. Figure 3.1 graphically depicts this relationship, which we refer to as Charnov *et al.*'s invariant rule. A decrease in between-clutch egg size variance was also predicted by Ebert (1994), though Charnov *et al.* (1995; Charnov & Downhower 1995) were the first to make the invariant predictions. The second invariant rule is that the increase in resources devoted to reproduction required to go from producing a clutch size of C to a clutch size of C+1 is invariant, regardless of clutch size (Downhower & Charnov 1998). We refer to this as Downhower and Charnov's invariant rule.

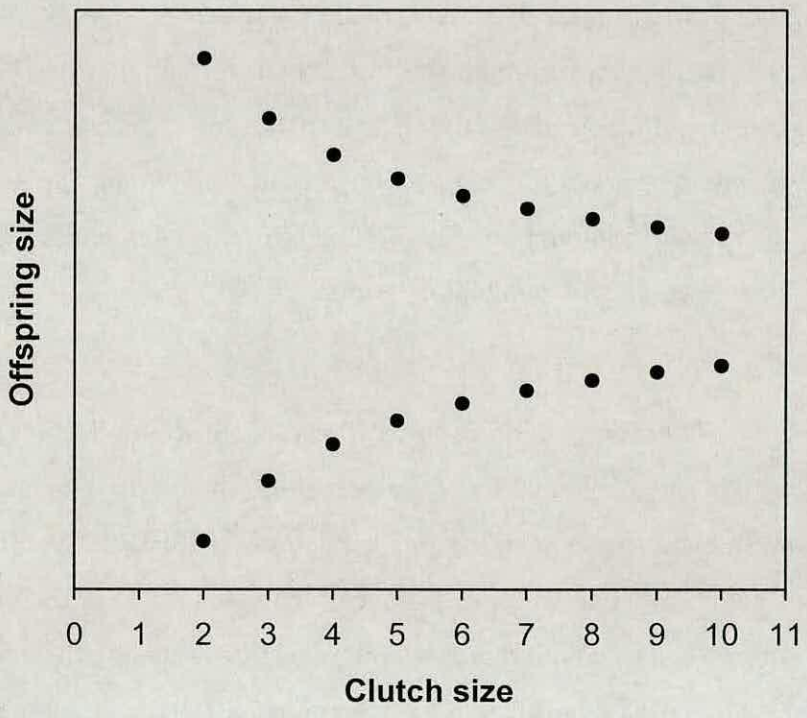

Figure 3.1: Predicted relationship between range in egg volume and clutch size (as described by Equation 3.1). The upper point refers to the largest egg volume and the lower point to the smallest egg volume predicted for that clutch size. The distance between the points represents the total range of egg sizes expected for that clutch size.

The usefulness of these two invariant rules is that they are independent of underlying trade-offs, such as the relationship between offspring size and fitness. This means that they can be tested quantitatively with relative ease in any organism that produces small (but variable) clutch sizes. Consequently, cases where the invariant rules do not hold quantitatively can suggest that the fundamental assumption of clutch size models do not hold. However, despite this possible wide applicability, there has yet to be a comprehensive, experimental test of these invariant predictions (but see Charnov *et al.* 1995; Mayhew 1998; Downhower & Charnov 1998; West *et al.* 2001).

Our aim here is to carry out an explicit experimental test of the predictions and assumptions of the invariant rules. We use a small, freshwater crustacean, *Daphnia magna*. This is an excellent model organism for testing the invariant rules because (a) clutch sizes can be in the relevant range (1-10); (b) we can experimentally manipulate, with extreme precision, the amount of resources a female has for reproduction by varying the amount of available food; (c) they are clonal, which allows us to compare genetically identical individuals.

We use egg volume as our measure of resource allocation, based on the fact that egg size correlates with eventual neonate size (Lampert 1993; Ebert 1994), and that egg size (or offspring size) correlates with fitness (Tessier & Consolatti 1989; Gliwicz & Guisande 1992), suggesting that larger eggs have more resources. We set out to maximise variation in egg size within each clutch size by maximising the variation in resources (food) within each clutch size. We accomplish this by having many food treatments and assigning only one *Daphnia* per treatment. Our statistical tests compare egg volumes between clutches of different sizes, not between food treatments. As *Daphnia* clutch size is correlated with food availability, we chose food treatments that would result in first clutches of between 1 and 10 offspring based on the results of a pilot study.

Our specific aims were to test whether: (1) variation in egg size decreases with increasing clutch size (Ebert 1994; Charnov *et al.* 1995; Charnov & Downhower 1995); (2) the range of egg sizes follows Charnov *et al.*'s invariant rule (1995; Charnov & Downhower 1995; Equation 3.1); (3) clutch size scales linearly with total resources devoted to reproduction as predicted by Downhower and Charnov's invariant rule (1998); and (4) whether resources are allocated equally within a clutch, which is an assumption implicit in all small clutch size models.

3.3 Materials and Methods

Study organism

We used a clone of *Daphnia magna* that had originated in the Gaarzerfeld pond, Northern Germany. *Daphnia magna* from this pond have been the focus of numerous studies of parasitism and life-history evolution (Little & Ebert 2001; Little *et al.* 2002). Throughout, *Daphnia* were fed on chemostat-grown cultures of the green algae *Scenedesmus sp.* and kept in incubators (20 C, 14:10 light/dark cycle). We raised three generations of *Daphnia* in jars containing 200mL *Daphnia* media, 5 *Daphnia* per jar with $1.5*10^7$ algal cells per day per jar (for 84 jars * 5 *Daphnia*/jar = 420 *Daphnia*) to equilibrate the conditions of the *Daphnia* and control for maternal and grand-maternal effects. For each new generation, we combined all neonates produced over a 24 hour period (first clutch neonates were not used) and randomly allocated neonates to jars. We changed *Daphnia* media every-other day. *Daphnia* media was a modified version of the Aachener Daphnien Medium described by Klüttgen *et al.* (1994).

Methods

We collected all offspring produced over a 15 hour period from the third generation (first clutch neonates were not used), placed them in separate jars containing 200mL

Daphnia media, and randomly assigned them to a food level. Food treatments ranged between $1.00 * 10^4$ and $3.50 * 10^6$ algal cells per day. Each treatment differed from the nearest food treatments by $1.00 * 10^4$ algal cells, resulting in 350 food treatments with one *Daphnia* per food treatment. The *Daphnia* jars were in trays, 12 jars to a tray. We randomised the location of the *Daphnia* within trays and the location of the trays within the incubator. We systematically rotated the jars within the trays, and the trays within the incubator each day. We changed the *Daphnia* media in all the jars every-other day. We checked the *Daphnia* twice per day, morning and evening, and recorded deaths. When the first clutch of eggs was produced, the mothers (still containing eggs) were photographed using a camera attached to a dissecting microscope. The eggs were then counted, removed and photographed. All eggs were in stage 1 of development as described by Threlkeld (1979). Later, the photographs were used to measure mother length (top of head to base of spine) and egg diameter. Since many of the eggs were oval in shape, both length (longest axis) and width (perpendicular across middle of length) of the eggs were measured. Egg volume was estimated by modelling the eggs as an ellipsoid shape as described by Equation 3.2.

$$Volume = \frac{4}{3} \times Length \times Width^2 \qquad (3.2)$$

Data was collected for only the first clutch of eggs.

Statistical analyses

We used the SAS System: Release 8.0 (SAS Institute) for all analyses not requiring resampling. Resampling was done in Microsoft Excel 2000 using the Poptools add-in (Hood 2002). When appropriate, we averaged egg volume over clutch to avoid pseudoreplication. We used generalised linear regression to test the relationship between clutch size and food level, and egg size and food level. Because variance

was heterogeneous, we used a Spearman's rank correlation to test whether egg volume (averaged over clutch) changed with clutch size.

We calculated the variance in egg volume for each clutch size (using egg volumes averaged over clutch) and used an ordered heterogeneity (OH) test to evaluate whether variance decreased with increasing clutch size. The OH test combines the *P*-value from a Bartlett's homogeneity of variance test ($P_{Bartlett}$) with the Spearman's rank correlation coefficient (r_s) to calculate the OH statistic (r_sPc) as follows: $r_sPc = r_s * (1-P_{Bartlett})$ (Rice & Gaines 1994).

We used data on the mean egg volume (averaged over clutch) to test whether range in egg volume decreased as predicted by Charnov *et al.*'s invariant rule (1995; Charnov & Downhower 1995). We tested Equation 3.1 for $\{i,j\} = \{2,1\}$ through $\{10,9\}$. We estimated the value of the invariant and the 95% confidence intervals for the left-hand side of Equation 3.1 by bootstrapping 10,000 resamples of egg volume for each clutch size, as described by West *et al.* (2001). As sample size affects range, we used the smaller sample size of the two clutch sizes, *i* and *j*, for both the numerator and denominator for each test.

Downhower and Charnov's invariant rule (1998) predicts that going from a clutch size of *C* to a clutch size of *C*+1 requires an invariant increase in resources allocated to reproduction irrespective of *C*. This would lead to a linear relationship between clutch size and resources devoted to reproduction. We tested this prediction by using generalised linear regression to relate clutch size to resources devoted to reproduction (number of eggs in a clutch multiplied by the mean egg volume for that clutch). We tested for non-linearity by determining the significance of adding a quadratic (squared) term to the model.

To test whether resources are allocated equally within clutches, we followed the randomisation procedure of West *et al.* (2001). Specifically, we calculated the mean within-clutch variance for each clutch size. Then, within each clutch size, we randomly allocated eggs to clutches, and calculated the mean within-clutch variance of our randomised clutches. We repeated this procedure 1000 times and tested whether the observed variance differed from the variance of our randomised clutches. This procedure was done separately for each clutch size. While there will always be some variation in size between eggs from the same clutch, this method allows us to compare the within-clutch variation to a measure of the possible variation in egg size. The sampling technique calculates the expected amount of variation assuming that egg sizes are randomly distributed. If the actual within-clutch variation is less than this calculated value based on the assumption of random distribution, this suggests that mothers are allocating resources (relatively) equally within a clutch.

3.4 Results

No reproduction was seen in animals receiving less than $2.70*10^5$ algal cells per day, nor in approximately 8% of *Daphnia* receiving more than this amount of food. We included only *Daphnia* that produced clutches of ≤10 eggs in analyses, as few *Daphnia* (<5%) produced >10 eggs. Our analyses contain data from 274 *Daphnia* and 1336 eggs. We observed the *Daphnia* for 21 days after being placed in their treatments, during which time all *Daphnia* had either produced a clutch of eggs or died (96% of those *Daphnia* that produced eggs did so within the first 14 days of the experiment). Clutch size increased with increasing food ($F_{1,272}$=534.18, P<0.0001; figure 3.2). Mean egg volume decreased with increasing food ($F_{1,259}$=89.2, P<0.0001; figure 3.3) and decreased with increasing clutch size (r_s=-0.34, P<0.0001, n=261; figure 3.4).

As predicted (Ebert 1994; Charnov *et al.* 1995; Charnov & Downhower 1995) variation in mean egg size (between clutches) decreased with increasing clutch size

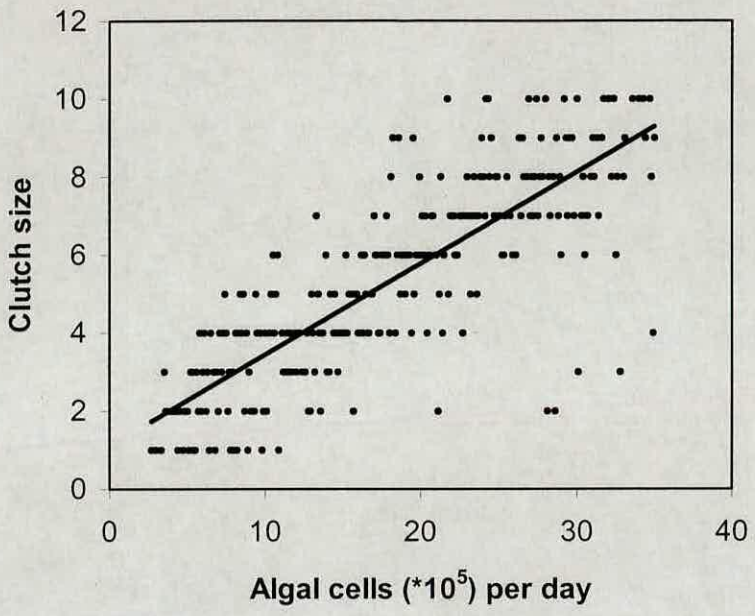

Figure 3.2: Relationship between food treatment and clutch size. Linear trend line: $y = 0.23x + 1.11$, $r^2 = 0.66$.

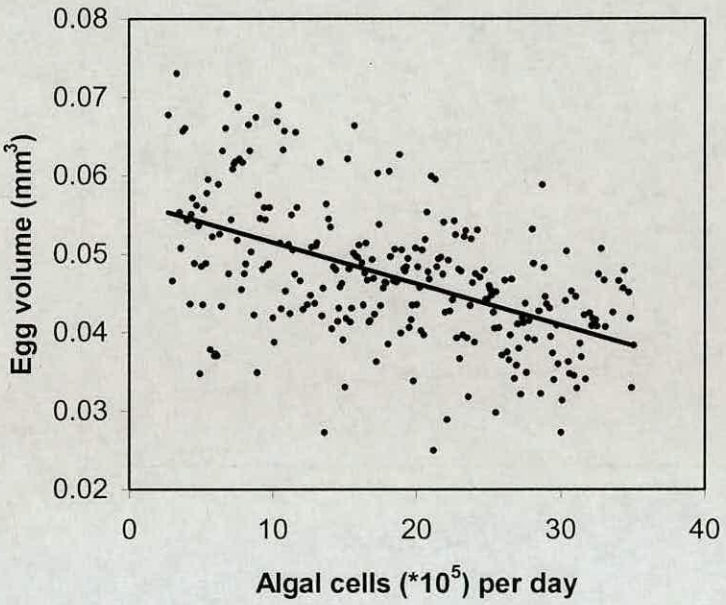

Figure 3.3: Relationship between food treatment and egg volume. Linear trend line: $y = -0.0005x + 0.057$, $r^2 = 0.26$.

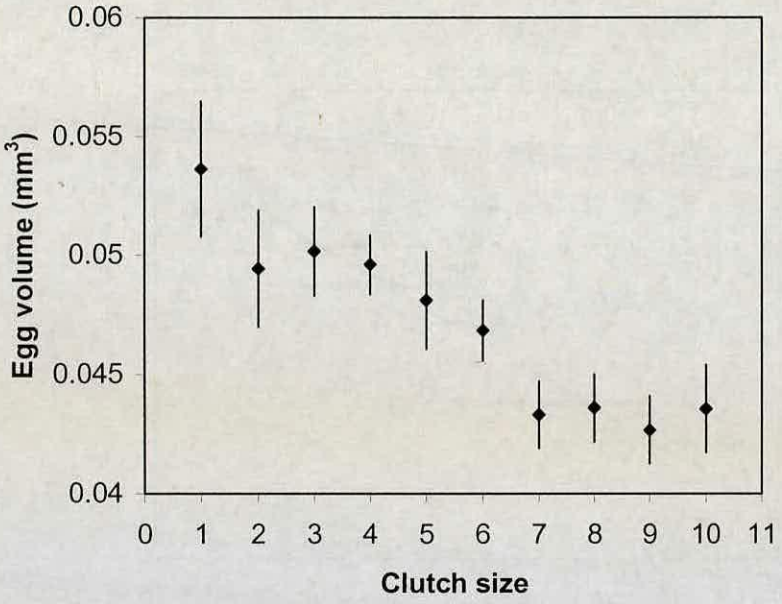

Figure 3.4: Mean egg volume for clutches of size 1-10. Bars are ±s.e.m.

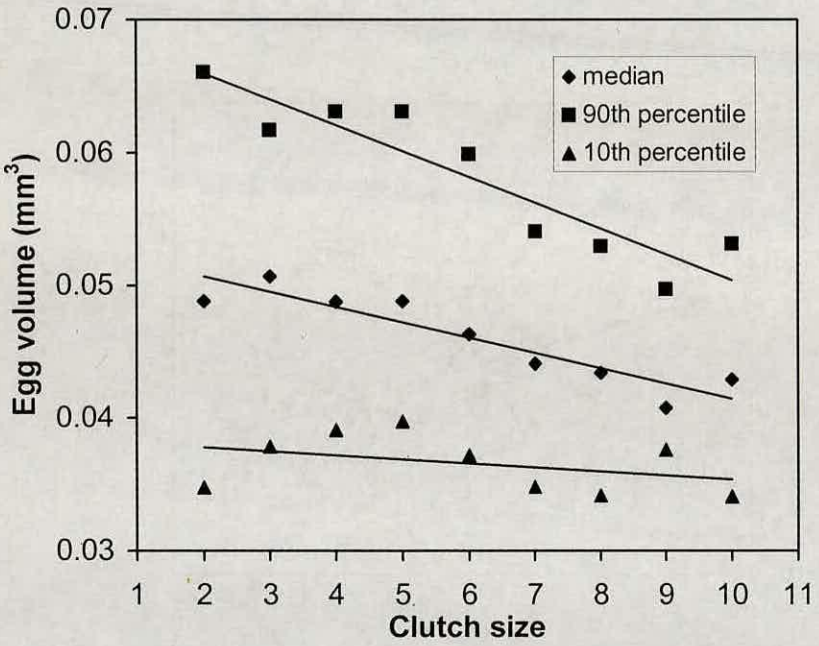

Figure 3.5: Median (diamonds), 90th percentile (triangles) and 10th percentile (squares) for egg volume for clutches of size 1-10. Linear trend line: Median: $y = -0.0011x + 0.053$, $r^2 = 0.85$; 90th percentile: $y = -0.0019x + 0.070$, $r^2 = 0.85$; 10th percentile: $y = -0.0003x + 0.038$, $r^2 = 0.15$.

(OH test: Spearman r_s value=-0.87, Bartlett *P*-value=0.11, r_sPc=-0.77, two-tailed *P*-value<0.001, figure 3.5). We used a random re-sampling technique to test the extent to which this led to a decrease in range of mean egg size with clutch size, as predicted by Charnov *et al.'s* invariant rule (1995; Charnov & Downhower 1995). The 95% confidence interval of the decrease in range included the invariant value predicted by Equation 1 for all clutch sizes tested (table 3.1). However, confidence intervals were large, and in all cases the 95% confidence intervals included 1. Consequently, when considering each pair of clutch sizes (i.e., comparing *C* to *C*+1), individually there was no statistically significant change in range. Overall, 6 of the 9 observed values were larger than the expected values. This lack of a close fit to Charnov *et al.'s* prediction is also shown by the fact that the range did not show a symmetrical convergence around the mean: whilst the 90th percentile of mean egg size significantly decreased with increasing clutch size (slope=-0.0019mm^3/clutch size, r_s=-0.92, n=10, *P*=0.0002), the 10th percentile did not significantly increase with increasing clutch size (slope=0.0003mm^3/clutch size; n=10, r_s=-0.61, *P*=0.060; figure 3.5).

Table 3.1: Test of Equation 3.1. The expected, observed (median of the 10,000 resamples) and 95 % confidence intervals for $(I_{max\_i}-I_{min\_i})/(I_{max\_j}-I_{min\_j})$ obtained by resampling the egg volume data (averaged over clutch).

Clutch sizes compared	Sample size	Expected C_{ji}/C_i	Observed $\frac{I_{max\_i}-I_{min\_i}}{I_{min\_j}-I_{min\_j}}$	Lower confidence limit (2.5%)	Upper confidence Limit (97.5%)
2 vs. 1	15	0.5	1.09	0.71	1.65
3 vs. 2	22	0.67	0.83	0.57	1.10
4 vs. 3	23	0.75	0.99	0.70	1.41
5 vs. 4	24	0.80	1.28	0.72	1.78
6 vs. 5	24	0.83	0.72	0.46	1.21
7 vs. 6	35	0.86	1.41	0.72	1.85
8 vs. 7	28	0.88	0.70	0.43	1.39
9 vs. 8	17	0.89	0.81	0.37	1.40
10 vs. 9	16	0.90	1.21	0.64	2.68

We carried out two further tests to ensure the robustness of the negative relationship between variance in egg volume and clutch size. First we tested that this pattern was not simply a statistical artefact due to averaging over more eggs as clutch size increased. To do this, we randomly chose one egg from each clutch, and calculated the regression line relating variance in egg volume to clutch size. We repeated this 100 times. All 100 regression lines had a negative slope, leading us to conclude that variance does indeed decrease with increasing clutch size ($P<0.01$). Second, we tested that the relationship did not arise merely due to differences in egg shape. We did this by testing for relationships between: (a) clutch size and egg length, and (b) the variance in egg length and clutch size. The results of both these tests were similar to those reported for egg volume above, supporting the robustness of our results.

Downhower and Charnov's invariant rule (1998) predicts that the amount of extra resources needed to produce an additional egg remains constant over clutch size. This therefore predicts a linear relationship between resources devoted to reproduction and clutch size. Resources devoted to reproduction was significantly related to clutch size (figure 3.6, $F_{1,259}=35.1$, $P<0.0001$), with an linear regression explaining 82.6% of the variation in the data. We then tested for non-linearity by adding a squared term (clutch size squared) to the model – the squared term did not significantly improved the model ($F_{1,258}=3.12$; $P=0.078$).

Clutch size models assume that each egg in a clutch receives equal resources. Our randomisation procedure allowed us to test this statistically, by comparing variation between eggs from the same clutch to that of eggs from different clutches (within the same clutch size). Eggs from the same clutch were more similar in volume than eggs from different clutches ($P<0.01$) for every clutch size tested (clutch sizes 2-10, table 3.2). While there will always be some variation in egg size within a clutch (otherwise column 3 of table 3.2 would only contain 0's), we've shown that, while the possible variation in egg size is quite large, mothers are producing clutches with

relatively similar sized eggs. This supports the hypothesis that mothers allocate resources relatively equally between eggs in a clutch.

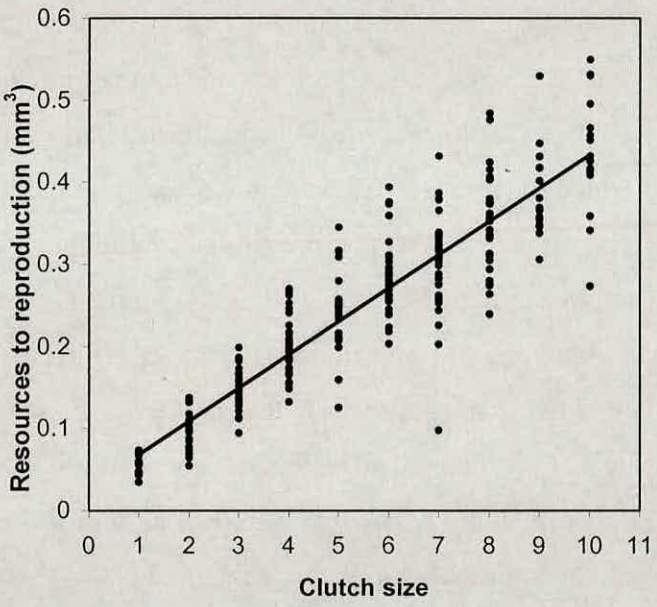

Figure 3.6: Relationship between clutch size and resources devoted to reproduction (number of eggs in a clutch multiplied by the mean egg volume for that clutch). Linear trend line included: $y = 0.041x + 0.028$; $r^2 = 0.83$).

Table 3.2: Observed mean within clutch variance in egg volume compared to sampled mean within clutch variance in egg volume by clutch size; 95% Confidence intervals are for sampled values.

Clutch size	Sample size	Obs. mean variance ($\times 10^{-5}$)	Sampled mean variance ($\times 10^{-5}$)	Lower Confidence limit (2.5%) ($\times 10^{-5}$)	Upper confidence limit (97.5%) ($\times 10^{-5}$)	*P*-value
2	22	2.81	11.25	6.46	16.18	<0.01
3	23	2.68	9.51	6.76	12.62	<0.01
4	45	2.65	7.87	6.58	9.34	<0.01
5	24	4.93	13.17	10.71	15.95	<0.01
6	35	4.55	8.27	6.15	10.28	<0.01
7	36	4.48	8.77	7.58	10.44	<0.01
8	28	4.61	8.82	7.66	10.40	<0.01
9	17	3.52	6.60	5.65	7.77	<0.01
10	16	3.47	8.19	7.41	9.06	<0.01

3.5 Discussion

We have carried out an experiment designed explicitly to test the predictions of small clutch models, and in particular their invariant predictions (figure 3.1; Ebert 1994; Charnov & Downhower 1995; Charnov *et al.* 1995; Downhower & Charnov 1998). In support of these invariants, we found that: (1) variance in egg volume decreased with clutch size (figure 3.5); and (2) clutch size showed a linear relationship to resources devoted to reproduction (figure 3.6). As assumed by these models, we found that: (3) eggs from the same clutch were more similar in volume than eggs from different clutches (within a clutch size), suggesting that mothers allocate resources relatively equally between eggs in a clutch (table 3.2). However: (4) mean egg volume decreased with clutch size (figure 3.4); and (5) the rate at which the range of egg volumes decreased with clutch size did not show a strong fit to the predictions of Charnov *et al.*'s invariant rule (1995; Charnov & Downhower 1995) regarding how the range of egg volumes should change with clutch size (table 3.1). Specifically, although the 95% confidence intervals of our data include the values predicted by Equation 3.1, the confidence intervals are large and in all cases include 1 (table 3.1). Moreover, the 10^{th} and 90^{th} percentiles of mean egg size per clutch did not converge symmetrically around the mean with increasing clutch size (figure 3.5). There are several possible hypotheses that could explain why range of egg sizes decreased more slowly than expected, which we discuss below.

First, there might exist a lower boundary on egg volume, below which eggs are not viable. A lower limit on egg size in *Daphnia* has been postulated previously (Glazier 1992) and, at small egg sizes, there is a strong positive correlation between egg size and likelihood of hatching (in *Daphnia pulex*; Bell 1983). The consequences of a minimum egg size is that in some circumstances, *Daphnia* females would reduce their clutch size by one rather than produce eggs smaller than this minimum egg volume, hence decreasing the possible egg volume/egg number combinations. Consistent with this, we found that, while the 90^{th} percentile of egg volumes

decreased with increasing clutch size, there was no change in the 10^{th} percentile (figure 3.5). A similar pattern has been observed in a fish (Charnov *et al.* 1995).

Second, the optimal egg size might vary with food and resource availability, and therefore clutch size. In our study, mean egg volume decreased with increasing clutch size. Decreasing egg (or neonate) size with increasing clutch size (or increasing food availability, as clutch size and food availability are correlated) has been found previously in *Daphnia* (Bradley *et al.* 1991; Gliwicz & Guisande 1992; Ebert 1993); although other patterns have also been observed (e.g. Lynch 1989; Tessier & Consolatti 1991; Glazier 1992; Boersma 1995). Equation 3.1 assumes that mean egg size remains constant. Our results are consistent with the hypothesis that mothers in bad environments (in this case, low food) produce larger eggs than mothers in good environments (high food). This would occur if the fitness benefit of being a large offspring is greater at lower food (Hutchinson 1951; Green 1966; Gliwicz & Guisande 1992).

Third, the relationship between resources allocated to an egg and egg volume might be non-linear. Egg volume is correlated with offspring fitness (as discussed in the Introduction), suggesting that larger eggs do indeed receive more resources than smaller eggs. However, the Carbon:Nitrogen ratios in neonates differ with the mothers' food availability (in *Daphnia pulicaria*; Tessier & Consolatti 1991), suggesting that resource provisioning may differ between eggs of different sizes. In addition, size can affect predation risk for *Daphnia*, and *Daphnia* can adjust age and size at maturity in line with predation risk (Stibor 1992; Weider and Pijanowska, 1993; Stibor & Lüning, 1994). In other organisms, complicated size-fitness relationships have been described, for example where fitness depends on an individual's size relative to competitors (Westoby *et al.* 1992; Rees and Westoby 1997).

Fourth, a female might be simultaneously optimising the amount of resources that she allocates to reproduction with the resources allocated elsewhere, such as to growth. If this is the case, then she might not be constrained to put a fixed amount into reproduction, dependent upon food levels.

Conclusions

Quantitative life-history predictions often require a detailed knowledge of difficult to measure life-history details. Recently, it has been shown that this problem can in some cases be resolved through the use of a dimensionless approach (Charnov 1993; Charnov 1997). This method can predict life-history invariants which permit quantitative tests in the absence of a detailed knowledge of underlying trade-offs and without the inclusion of biological complexities. As well as the small clutch size invariants tested here, life-history invariants have been successfully utilised to explore topics as varied as age at maturity in parasitic nematodes (Gemmill *et al.* 1999), timing of sex change in fish (Charnov & Skuladottir 2000; Allsop & West 2003a; Allsop & West 2003b), and life-history characteristics (age at maturity, cellular maintenance rate) in mammals (Charnov 2001).

In this study, Charnov *et al.*'s invariants (1995; Charnov and Downhower 1995; Downhower and Charnov 1998) have proven useful for expanding our knowledge specifically about *Daphnia* reproduction, and generally about resource investment into offspring. We found qualitative support for the prediction that variance in egg volume should decrease with increasing clutch size (Ebert 1994; Charnov & Downhower 1995; Charnov *et al.* 1995; figure 3.5). Where the data do not fit the invariant predictions quantitatively has lead to several hypotheses about *Daphnia* reproduction, and about resource allocation in general, highlighted above. These observations require further investigation. In particular: Is there a minimum viable egg volume? Does the relationship between egg volume and offspring fitness vary with food availability, and thus clutch size? To what extent are our findings generalisable to more species?

CHAPTER 4

TESTING PREDICTIONS OF SMALL BROOD MODELS USING PARASITOID WASPS

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4.1 Abstract

Life-history theory makes testable predictions for how the mean and variance in offspring size should change with brood (litter) size. We use previously collected data on six parasitoid wasp species to test the predictions and assumptions of these models. We found support for the qualitative prediction that variance in offspring size among broods should decrease with increasing brood size. In contrast, we did not find consistent support for the quantitative predictions of how variance in offspring size should decrease with increasing brood size. We also did not find consistent support for the prediction that the range of resources devoted to reproduction by different individuals producing a brood of a particular size should be constant over brood sizes. The assumption that mean offspring size is constant over brood size was violated in three of six species. The assumption that resources are shared equally between individuals within a brood generally held. We discuss possible explanations where our results did not fit the predictions of theory.

4.2 Introduction

The trade-off between offspring size and offspring number is a classic problem in evolutionary biology (Smith & Fretwell 1974; Schaffer & Gadgil 1975; Parker & Begon 1986; Godfray *et al.* 1991; Stearns 1992), but we still do not have a complete understanding of how and why offspring size varies among litters. The classic approach to this problem, as described by Smith and Fretwell (1974), predicts optimum offspring size by maximizing clutch (and therefore maternal) fitness, and this approach has been widely utilized to explore offspring size. However, the predicted number of offspring is generally not a whole-number, while the number of offspring produced by an organism is necessarily an integer value.

This integer problem has recently led to several theoretical models exploring offspring size in small broods (Ebert 1994; Charnov *et al.* 1995; Charnov & Downhower 1995; Downhower & Charnov 1998). These small brood models make several predictions about how the mean and range in offspring size is related to brood size. Here, we test 3 of the predictions, and 2 of the assumptions, of these models. Prediction 1 states that the range in offspring size (between different broods) should decrease with increasing brood size (Ebert 1994; Charnov & Downhower 1995; Charnov *et al.* 1995). Prediction 2 describes this decrease, whereby the decrease in the range in offspring size is proportional to the brood size (Charnov & Downhower, 1995; Charnov *et al.* 1995; West *et al.* 2001). This is given formally by:

$$\frac{I_{\max\_i} - I_{\min\_i}}{I_{\max\_j} - I_{\min\_j}} = \frac{C_j}{C_i} \qquad (4.1)$$

where I is offspring size, C is brood size, $I_{max\,i}$ and $I_{min\,i}$ are the maximum and minimum size of offspring in broods of C_i young, i and j are broods of different

sizes, and $i=j+1$. Figure 4.1 graphically depicts this relationship, which we refer to as Charnov *et al.*'s invariant rule (1995; Charnov & Downhower 1995).

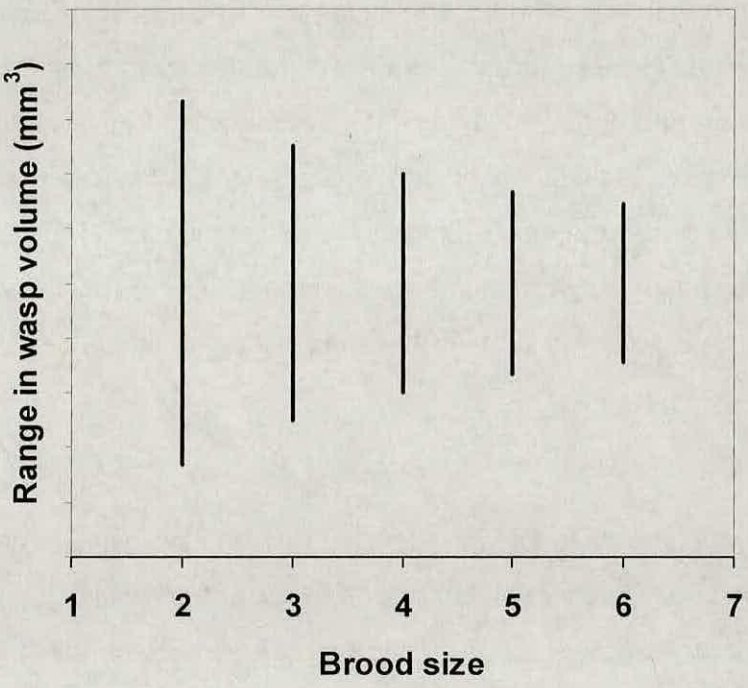

Figure 4.1: Range in wasp body size and brood size as predicted by Charnov *et al.*'s invariant and described by Equation 4.1. The solid line at each brood size represents the range in body size for that brood size.

Prediction 3 states that the range in resources put towards reproduction (for mothers producing a certain brood size) should be invariant over brood size (Downhower & Charnov 1998). Put another way, the relationship between total resources devoted to a brood and brood size should be linear. We refer to this as Downhower and Charnov's invariant rule (1998).

The study reported here is the first to test these invariants using multiple species, as we utilize previously published data on six parasitoid wasp species. The advantage of using published data is that it allows predictions to be tested in multiple species and ecological systems, and hence the generality of the models can be examined.

Parasitic wasps are ideal for testing these models as there are a number of species that produce small, but variable, sized broods (Godfray 1994; Mayhew & Hardy 1998). The host organism provides all the resources for the wasp offspring until they mature to adulthood. Resources (measured as body size) and fitness are correlated in parasitoids (van den Assem *et al.* 1989; Hardy *et al.* 1992; Visser 1994; Godfray 1994; West *et al.* 1996; Petersen & Hardy 1996). We would expect wasp offspring to follow the invariant rules described above if: (1) female wasps can accurately assess host size (resources available to the developing brood) and adjust brood size accordingly; (2) average resources per offspring is constant over brood size; and (3) resources are shared equally between members of a brood. There is much evidence for assumption (1), reviewed by Godfray (1994), and we test assumptions (2) and (3) here.

Our specific aims are to test the predictions that: (1) variation in wasp size decreases with increasing brood size (Ebert 1994; Charnov & Downhower 1995; Charnov *et al.* 1995); (2) the range of wasp sizes follows Charnov *et al.*'s invariant rule (1995; Charnov & Downhower 1995; Equation 4.1; figure 4.1); and (3) brood size scales linearly with resources devoted to reproduction as predicted by Downhower and Charnov's invariant rule (1998). We also test two assumptions made by the small brood size models, that: (4) mean offspring size is constant over brood size; and (5) resources are divided equally within a brood.

4.3 Materials and Methods

Study organisms

We collated data from wasp researchers that allowed us to test the above predictions from parasitoid wasps that produce small, but variable, brood sizes. We located potentially useful studies by searching reviews of the subject (e.g. Godfray 1994), and by directly contacting wasp researchers. Once potentially useful studies where located, we contacted the original authors in order to obtain the necessary raw data.

Below, we briefly describe the species of wasps included in this study. We obtained data on six species, and the type of data (field or experimental), sample sizes and how wasp body size was measured are summarised in table 4.1. Detailed information on the biology of each wasp species, materials and methods are provided in the original publications. For two of the wasp species (*Achrysocharoides zwoelferi* and *Laelius pedatus*) the original publications contain similar and/or identical analyses testing some of the same hypotheses we test here. However, they are included here because we are looking for patterns that generalise over multiple species, and because we needed to standardise methods of analysis across species.

Table 4.1: Background information for each dataset.

Species	Source of data	Type of data	Body size measure	N (# broods)	BS a. mean b. range	% F	Are F or M larger?	Sex	Change in body size with BS?
A. zwoelferi	West *et al.*, 1996; 2001	Field	HTL^3	162	a. --- b. 1-4	---	†F**	F M	†↓** ---
A. pallipes	Mayhew and van Alphen, 1999	Lab	HTL^3	33	a. 2.31 b. 1-5	19%	NS	F M	--- ↓*
E. cacoeciae	Fidgen *et al.*, 2000	Lab and exp field	Pupal L^3	441	a. 3.20 b. 1-18	55%	F****	F M	↓NS ↑NS
L. pedatus	Mayhew, 1998	Lab	Mesosoma length^3	164	a. 2.15 b. 1-5	68%	F****	F M	↑NS ↑NS
M. ridibundus	Bezemer and Mills, 2003	Lab	HTL^3	358	a. 3.18 b. 1-9	50%	F****	F M	↓**** ↓****
M. stanleyi	Bernal *et al.*, 1999	Lab	HTL^3	395	a. 1.67 b. 1-10	44%	F****	F M	↓NS ↓NS

M = male; F = female; BS = brood size; HTL = hind tibia length, 'Experimental field' = data from experiments that were carried out in the field but manipulated by the researchers, e.g. wasps were released into the area or hosts were placed on trees. Significance levels: NS $P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$; †Result first reported in original source; --- indicates not applicable or sample size too small to test. Note: As we are looking for general patterns in the data, we sometimes average over factors (such as host instar number, or host size) that might affect mean brood size, % female, and change in body size with BS. Therefore results reported here do not necessarily reflect the patterns expected in nature, and are only included to give an overall understanding of the data.

Achrysocharoides zwoelferi (Hymenoptera: Eulophidae) attacks members of the species *Phyllonorycter* that mine the leaves of *Salix* spp. Data reported here are both observational and experimentally manipulated field data with *Phyllonorycter salicicolella* as hosts (West *et al.* 1996; West *et al.* 2001). *Metaphycus stanleyi* (Hymenoptera: Encyrtidae) is a parasitoid of many soft scales. Reported here are laboratory data with brown soft scales as hosts (Bernal *et al.* 1999). *Aphaereta pallipes* (Hymenoptera: Braconidae) is a parasitoid of cyclorrhaphous Diptera.

Reported here are laboratory data using *Drosophila virilis* as hosts (Mayhew & van Alphen 1999). *Laelius pedatus* (Hymenoptera: Bethylidae) is a parasitoid of the larvae of dermestid beetles. Data are from the laboratory with larvae of the beetle *Anthrenus flavipes* as hosts (Mayhew 1998). *Elachertus cacoeciae* (Hymenoptera: Eulophidae) attacks at least 13 species of Lepidoptera (Schauff 1985; Huber *et al.* 1996). Data are experimentally manipulated field data with spruce budworm (*Choristoneura fumiferana*) larvae as hosts (Fidgen *et al.* 2000). *Mastrus ridibundus* (Hymenoptera: Ichneumonidae) is a parasitoid of codling moth cocoons (*Cydia pomonella*). Data are from the laboratory using codling moth cocoons as hosts (Bezemer & Mills 2003). For all data, brood size is the defined as the number of young found in/on, or emerging from, the host.

Statistical analyses

All reported *P*-values are for two-tailed tests, using the 0.05 level of significance. We calculated the sex ratio (% female) by dividing the total number of female wasps by the total number of wasps, irrespective of natal brood. We tested whether body size differed by sex for each wasp species using a mixed model analysis with the model equation: wasp size = sex + brood size with brood identity included as a random factor. Tests for sex ratio, OH tests, and tests of Downhower and Charnov's invariant rule were carried out using the SAS System: Release 8.0 (SAS Institute). Tests for Charnov *et al.'s* invariant rule and sampling within broods were carried out using Microsoft Excel 2000 and the Poptools add-in (Hood 2002).

Variation in body size and brood size—We used an ordered heterogeneity (OH) test to evaluate whether variance in mean body size (averaged over brood, by sex) decreased with increasing brood size. The OH test combines the *P*-value from a Bartlett's homogeneity of variance test (Pc) with the Spearman's rank correlation coefficient (r_s) to calculate the OH statistic (r_sPc) as follows: $r_sPc = r_s \times (1-P_{Bartlett})$ (Rice & Gaines 1994). We used wasp size averaged over brood (by sex) to avoid pseudo-replication (Hurlbert 1984).

Charnov et al. *'s invariant rule*—We used data on mean size of male and female wasps to determine if the range of wasp body sizes decreased with increasing brood size as predicted by Charnov *et al.*'s invariant rule (1995; Charnov & Downhower 1995). We estimated the value of the invariant and the 95% confidence intervals by bootstrapping 10,000 re-samples of the left-hand side of Equation 4.1, as described in West *et al.* (2001). As sample size affects range, we used the smaller sample size of the two brood sizes, *i* and *j*, for both the numerator and denominator for each test. This was only carried out when $n \geq 5$ for both *i* and *j*. We include brood sizes of 1 in our analyses, although it is more difficult to make precise predictions about variance when brood size is 1 than at other brood sizes (Charnov *et al.* 1995; Charnov and Downhower 1995).

Downhower and Charnov's invariant rule—We tested whether the resources necessary to increase from a brood size of *C* to a brood size of *C*+1 is constant over brood size as predicted by Downhower and Charnov's (1998) invariant rule. To do this, we used generalised linear regression to determine if brood size was related positively to host size. We tested for goodness of fit to non-linear models by testing the significance of adding a squared and a cubic term (model equations are outlined in table 4.3). We use host size in mg or length^3 as the measure of available resources.

Mean body size and brood size—Wasp sizes were first averaged over brood. Because variance was heterogeneous for many of the species tested, we used Spearman's rank correlations to test whether body size changed with brood size.

Are resources shared equally within a brood? —We tested whether resources were allocated equally within a brood by following the randomisation procedure of West *et al.* (2001). Specifically, we calculated the average within brood variance for each sex and brood size combination where $n \geq 5$. We then randomly allocated wasps to broods, calculated the within brood variance of our randomised broods, and repeated this procedure 10,000 times. We then compared the observed within-brood variance to the sampled values. This procedure was carried out separately for each brood size.

4.4 Results

We collated previously-published data from 6 species of wasp. Four species include data on male and female wasps. The *A. zwoelferi* dataset contains data for females only. The *A. pallipes* dataset does not contain enough females for analyses on female wasps. For all species except *A. pallipes*, females were significantly larger than males (table 4.1; $P<0.01$). Therefore all subsequent analyses were carried out separately for males and females.

Variation in body size and brood size—For every species/sex combination, a general pattern of decreasing variance (between broods) with increasing brood size was observed (figure 4.2), confirmed by negative r_s values, but not all were statistically significant (table 4.2). It is highly unlikely that we would find the general pattern of decreasing variance with increasing brood size in all combinations studied by chance (two-tailed sign test, n=10, $P<0.01$). The Ordered Heterogeneity (OH) test was statistically significant for 4 of the 10 species/sex combinations tested (table 4.2). The OH test includes all brood sizes that contained at least two broods. To ensure that brood sizes containing few broods were not unduly affecting our results, we also

carried out the OH test analyses only including brood sizes with ≥ 5 broods. Results were similar with the differences being that one analysis became statistically significant (*L. pedatus* females), two analyses became (not significantly) positive (*L. pedatus* males and *M. ridibundus* females) and one analysis showed no relationship (*E. cacoeciae* females).

In addition, using a random re-sampling technique, we found that the decrease in between-brood variance with brood size was, in general, slower than predicted. The ratio $C/C+1$ (Equation 4.1) was generally ≤ 1 (where the ratio is ≤ 1 for over half of all C's tested for that species/sex combination) in all five species tested for females (17 of 21 brood size/species combinations, two-tailed sign test, $P<0.01$; table 4.2). For males this was only true for one of five species (10 of 21 brood size/species combinations, two-tailed sign test, $P>0.10$; table 4.2).

Charnov et al.*'s invariant rule*—The observed ratio of C to $C+1$ was generally greater than the predicted ratio for both females (4 of 5 species; 19 of 21 brood size/species combinations, two-tailed sign test, $P<0.01$; table 4.2) and males (4 of 5 species; 17 of 21 brood size/species combinations, two-tailed sign test, $P<0.01$; table 4.2). The 95% confidence interval of the decrease in range included the invariant value predicted by Equation 4.1 in most cases for both females (4 of 5 species, 15 of 21 brood size/species combinations; table 4.2) and males (4 of 5 species; 14 of 21 brood size/species combinations; table 4.2). However, confidence intervals were large, and in most cases the 95% confidence intervals included 1 for both females (4 of 5 species; 16 of 21 brood size/species combinations; table 4.2) and males (4 of 5 species; 19 of 21 brood size/species combinations; table 4.2). Consequently, when considering each pair of brood sizes (i.e., comparing C to $C+1$), individually there was no statistically significant change in range.

Downhower and Charnov's invariant rule—We tested Downhower and Charnov's invariant rule (1998) by looking for a linear relationship between resources devoted

to reproduction and brood size. Brood size increased with increasing host size in all species tested using the linear model (table 4.3; figure 4.3). For one of the three species tested (*A. pallipes*), no quadratic terms were statistically significant, while for two species (*L. pedatus* and *M. stanleyi*) the squared term was significant. The cubic term was not significant for any species ($P>0.10$). Where the squared term was significant, it appeared that brood size increased rapidly with host size at small host sizes, but a few hosts contained broods that were much larger than the average for that host size.

Mean body size and brood size—For three wasp species (*A. zwoelferi* females; *A. pallipes* males; and *M. ridibundus* males and females), wasp size decreased with increasing brood size (table 4.1). For the other three species tested, wasp size did not vary (significantly) with brood size.

Are resources shared equally within a brood? —Our results support the assumption made by small brood size models that each individual in a brood receives equal resources. Our randomisation procedure allowed us to test this statistically, by comparing variation in body size of wasps from the same brood to that of wasps from different broods (within the same brood size). The 95% confidence intervals obtained from our sampling procedure generally excluded the observed value for mean variance for 3 of 5 species for females (12 of 20 brood size/species combinations, two-tailed sign test, $P>0.10$; table 4.4) and 2 of 4 species for males (10 of 17 brood size/species combinations, two-tailed sign test, $P>0.10$; table 4.4). In addition, in most cases, predicted variance was greater than observed variance (females: 4 of 5 species, 18 of 20 brood size/species combinations, two-tailed sign test, $P<0.01$; males: 3 of 4 species, 15 of 17 brood size/species combinations, two-tailed sign test, $P<0.01$; table 4.4). Some variation in offspring size will naturally occur within broods (see column 6; table 4.4). Our analysis has shown that, while the variation in wasp size in the population is relatively large, mothers are producing broods of (relatively) similar sized offspring, much more so than if offspring size

were randomly distributed in broods. This suggests that mothers allocate resources relatively equally between offspring in the same brood.

Figure 4.2: 90^{th} percentile (triangles), Median (squares) and 10^{th} percentile (circles) wasp volumes for each of the species tested, separately by sex. For simplicity, only brood sizes up to 6 are included. Linear trend lines are included.

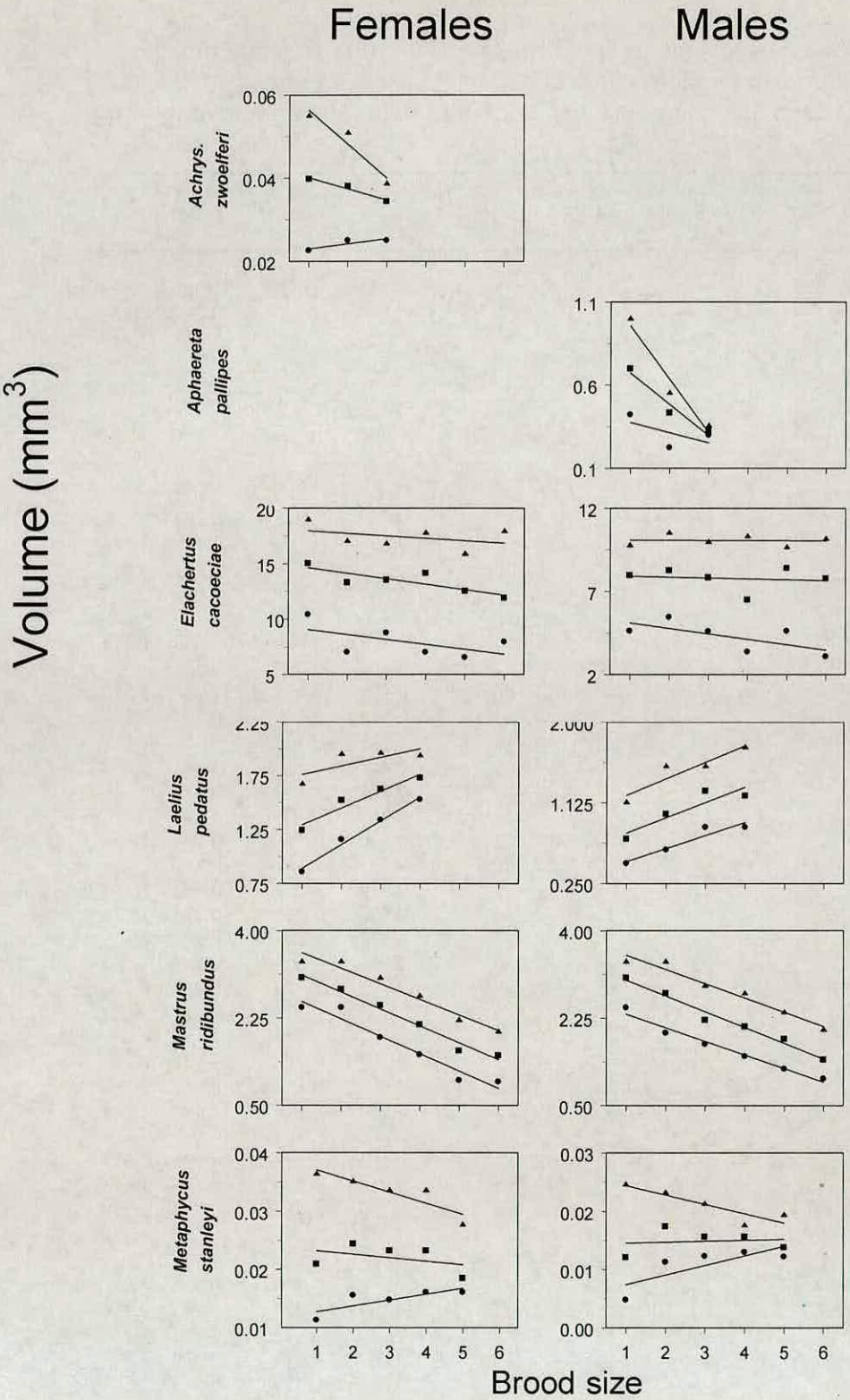
Females
Males
Volume (mm3)
Achrys. zwoelferi
Aphaereta pallipes
Elachertus cacoeciae
Laelius pedatus
Mastrus ridibundus
Metaphycus stanleyi
Brood size

Table 4.2: Results of OH test and test of Equation 4.1. The expected, observed (mean of the 10,000 re-samples) and 95% confidence intervals for $(l_{max\_i}-l_{min\_i})/(l_{max\_j}-l_{min\_j})$ obtained by re-sampling the wasp body-size data (averaged over brood).

Species	Source	Sex	How variance changes with BS	BS	N	Exp.	Obs. (mean)	Confidence Interval
A. zwoelferi	West *et al.* 1996; 2001	F	↓****, r_s = -1.00, Pc<0.0001, k=3	2 vs 1	60	0.500	†0.759	†0.601 – 0.954
				3 vs 2	35	0.667	†0.503	†0.358 – 0.681
		M	---	---	---	---	---	---
A. pallipes	Mayhew and van Alphen 1999	F	---	---	---	---	---	---
		M	↓****, r_s = -1.00, Pc=0.0011, k=3	2 vs 1	6	0.500	1.011	0.257 – 4.804
				3 vs 2	10	0.667	0.392	0.149 – 0.828
E. cacoeciae	Fidgen *et al.* 2000	F	↓ NS, r_s = -0.10, Pc=0.88, k=10	2 vs 1	45	0.500	0.951	0.696 – 1.392
				3 vs 2	34	0.667	0.693	0.532 – 0.900
				4 vs 3	30	0.750	1.425	1.057 – 1.832
				5 vs 4	17	0.800	0.809	0.598 – 1.267
				6 vs 5	17	0.833	0.966	0.761 – 1.133
				7 vs 6	10	0.857	0.916	0.423 – 1.565
				8 vs 7	8	0.875	1.374	0.639 – 2.584
		M	↓ NS, r_s = -0.15, Pc=0.87, k=10	2 vs 1	55	0.500	0.788	0.598 – 1.138
				3 vs 2	40	0.667	1.152	0.849 – 1.454
				4 vs 3	27	0.750	1.102	0.793 – 1.585
				5 vs 4	22	0.800	0.896	0.578 – 1.253
				6 vs 5	18	0.833	1.068	0.666 – 1.685
				7 vs 6	7	0.857	0.806	0.184 – 1.794
				8 vs 7	5	0.875	1.120	0.227 – 2.329
L. pedatus	Mayhew 1998	F	↓ NS, r_s = -0.40, Pc=0.05, k=5	2 vs 1	40	0.500	0.792	0.561 – 1.340
				3 vs 2	36	0.667	0.886	0.657 – 1.150
				4 vs 3	20	0.750	0.849	0.406 – 1.305
		M	↓ NS, r_s = -0.30, Pc=0.18, k=5	2 vs 1	20	0.500	1.332	0.895 – 2.023
				3 vs 2	34	0.667	0.818	0.606 – 1.014
				4 vs 3	20	0.750	1.224	0.782 – 1.918
M. ridibundus	Bezemer and Mills 2003	F	↓ NS, r_s = -0.26, Pc=0.086, k=8	2 vs 1	20	0.500	1.340	0.931 – 1.867
				3 vs 2	60	0.667	1.659	1.170 – 2.158
				4 vs 3	49	0.750	0.875	0.654 – 1.212
				5 vs 4	33	0.800	0.834	0.619 – 1.093
				6 vs 5	8	0.833	0.668	0.384 – 1.581
		M						
			↓ NS, r_s = -0.54, Pc=0.77, k=7	2 vs 1	15	0.500	1.264	0.755 – 2.037
				3 vs 2	50	0.667	1.150	0.730 – 1.654
				4 vs 3	56	0.750	1.104	0.640 – 1.615
				5 vs 4	37	0.800	0.719	0.491 – 1.097
				6 vs 5	10	0.833	0.854	0.510 – 1.345

M. stanleyi	Bernal *et al.*, 1999	F	↓****, r_s = -1.00, Pc=0.019, k=8	2 vs 1	60	0.500	0.862	0.009 – 1.026
				3 vs 2	23	0.667	0.883	0.065 – 1.252
				4 vs 3	12	0.750	0.846	0.611 – 1.280
				5 vs 4	7	0.800	0.617	0.155 – 1.219
		M	↓**, r_s = -0.71, Pc<0.0001, k=8	2 vs 1	60	0.500	0.630	0.416 – 0.896
				3 vs 2	21	0.667	0.907	0.489 – 1.417
				4 vs 3	14	0.750	0.630	0.268 – 1.130
				5 vs 4	7	0.800	1.280	0.240 – 3.264

M = male; F = female; BS = brood size. Significance levels: NS $P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$; r_s is the Spearman's rank correlation coefficient; Pc is the *P*-value from a Bartlett's homogeneity of variance test; k is the number of groups; [†]Result first reported in original source; --- indicates not applicable or sample size too small to test. The number of brood sizes included in the OH test (k) is sometimes greater than the number of brood sizes tested directly compared (columns 5-9) because all brood sizes with ≥ 2 brood were included in the OH test while only brood sizes with ≥ 5 broods are included in columns 5-9.

Table 4.3: Relationship between host size and brood size.

Species	Source	Host size measure	HS = BS Best fit equation, r^2, *F*-stat and *P*-value for highest order term	HS = BS + BS^2 Best fit equation, r^2, *F*-stat and *P*-value for highest order term
A. pallipes	Mayhew and van Alphen 1999	$Length^3$	HS = 7.58 BS + 66.03 r^2 = 0.19 BS: $F_{1,30}$ = 6.8, *P*=0.014	HS = -2.51 BS^2 + 20.47 BS + 51.74 r^2 = 0.22 BS^2: $F_{2,29}$ = 1.4, *P*=0.24
L. pedatus	Mayhew 1998	mg	HS = 1.37 BS - 0.28 r^2 = 0.68 BS: $F_{1,162}$ = 136.7, *P*<0.0001	HS = -0.05 BS^2 +1.62 BS – 0.52 r^2 = 0.68 BS^2: $F_{2,161}$ = 7.0, *P*=0.0088
M. stanleyi	Bernal *et al.* 1999	$Length^3$	HS = 3.12 BS + 5.34 r^2 = 0.22 BS: $F_{1,393}$ = 109.6, *P*<0.0001	HS = -0.44 BS^2 + 6.56 BS + 1.63 r^2 = 0.25 BS^2: $F_{2,392}$ = 19.1, *P*<0.0001

M = male; F = female; BS = brood size, HS = host size.

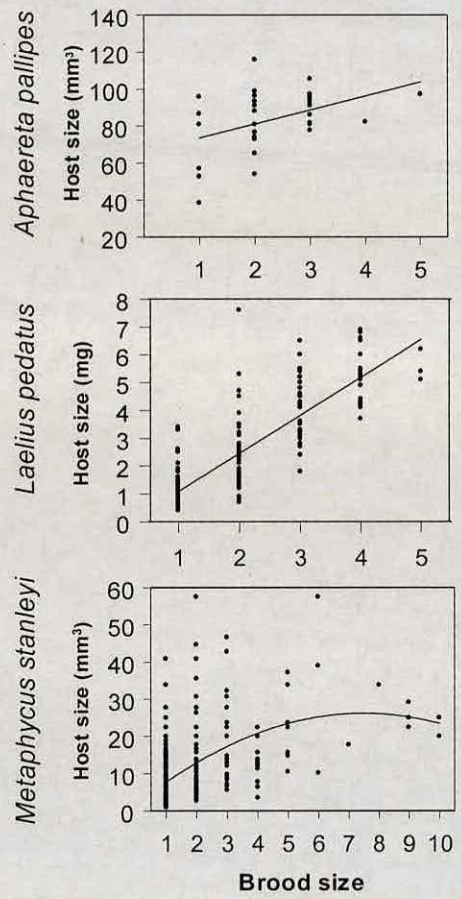

Figure 4.3: Relationship between brood size and host size. Best fit lines are included. See Table 4.3 for trend line equations and r^2 values.

4.5 Discussion

We analysed previously reported data on parasitoid wasps to test the predictions of small brood (litter) size models, and in particular their invariant rules (Ebert 1994; Charnov & Downhower 1995; Charnov *et al.* 1995; Downhower & Charnov 1998; West *et al.* 2001). The predictions/assumptions tested and our findings are summarised as follows:

(1) Our findings support prediction 1 that variance in offspring size among broods decreases with increasing brood size (figure 4.2; table 4.2).

(2) In general, our findings agree with the prediction of Charnov *et al.*'s invariant rule (1995; Charnov & Downhower 1995) of decreasing variance in offspring size with increasing brood size, but the observed decrease was slower than predicted (figure 4.2; table 4.2).

(3) Our findings do not agree with the predictions of Downhower and Charnov's invariant rule (1998), as brood size showed a non-linear relationship to available resources for 2 of 3 species tested (figure 4.3; table 4.3).

(4) Generally, our findings do not agree with assumption 1 that offspring size is constant over brood size. This assumption was violated in half (3 of 6) of the species tested in this study. For those species that violated this assumption, offspring size decreased with increasing brood size (table 4.1).

(5) Our results support assumption 2 that resources are divided equally within a brood (table 4.4).

We used Charnov *et al.*'s invariants (1995; Charnov and Downhower 1995; Downhower and Charnov 1998) to expand our knowledge specifically about the relationship between brood size and offspring size. Charnov and Downhower (1995) have calculated that Charnov *et al.*'s invariant rule should hold in a variety of animals with a variety of offspring size/offspring fitness functions. Previous studies

Table 4.4: Observed mean within brood variance in wasp body size volume compared to sampled mean within brood variance in wasp body size volume by brood size; 95% Confidence intervals are for sampled values.

Species	Source	Sex	BS	N	Obs. (mean) variance	Sampled (mean) variance	95% Confidence Interval
A. zwoelferi	West *et al.* 1996; 2001	F	2	34	†3.7×10^{-5}	†4.5×10^{-4}	†$2.7 \times 10^{-4} - 6.6 \times 10^{-4}$
		M	---	---	---	---	---
A. pallipes	Mayhew and van Alphen 1999	F	---	---	---	---	---
		M	2	7	0.00927	0.01952	0.00520 – 0.04018
			3	10	0.00456	0.00411	0.00193 – 0.00683
E. cacoeciae	Fidgen *et al.* 2000	F	2	28	4.088	12.500	7.152 – 18.856
			3	28	5.890	11.265	6.503 – 17.345
			4	26	2.628	12.250	7.775 – 17.734
			5	16	3.659	13.024	8.239 – 18.757
			6	18	3.986	13.539	10.097 – 17.627
			7	8	6.405	8.706	5.487 – 12.646
			8	8	5.900	17.749	12.342 – 24.055
		M	2	65	1.173	3.837	2.647 – 5.203
			3	37	2.066	5.947	4.198 – 7.948
			4	24	1.953	6.024	4.067 – 8.376
			5	18	2.724	3.653	2.467 – 4.988
			6	15	2.901	4.839	3.285 – 6.593
			7	6	2.207	4.805	2.586 – 7.996
L. pedatus	Mayhew 1998	F	2	9	0.0515	0.0895	0.0261 – 0.1800
			3	36	0.0527	0.0929	0.0502 – 0.1496
			4	20	0.0518	0.0672	0.0299 – 0.1246
		M	---	---	---	---	---
M. ridibundus	Bezemer and Mills 2003	F	2	31	0.157	0.198	0.108 – 0.322
			3	59	0.197	0.292	0.201 – 0.404
			4	39	0.172	0.324	0.228 – 0.436
			5	27	0.107	0.227	0.137 – 0.344
			6	7	0.121	0.236	0.108 – 0.430
		M	2	21	0.050	0.192	0.096 – 0.318
			3	57	0.252	0.286	0.206 – 0.381
			4	36	0.079	0.201	0.137 – 0.279
			5	33	0.104	0.244	0.170 – 0.332
			6	8	0.049	0.153	0.090 – 0.235
M. stanleyi	Bernal *et al.* 1999	F	2	15	5.6×10^{-5}	5.1×10^{-5}	$2.1 \times 10^{-5} - 9.5 \times 10^{-5}$
			3	13	2.7×10^{-5}	6.8×10^{-5}	$3.5 \times 10^{-5} - 10.8 \times 10^{-5}$
			4	9	1.9×10^{-5}	5.6×10^{-5}	$2.3 \times 10^{-5} - 9.5 \times 10^{-5}$
			5	7	4.2×10^{-5}	3.8×10^{-5}	$1.4 \times 10^{-5} - 7.2 \times 10^{-5}$
		M	2	15	2.4×10^{-5}	2.6×10^{-5}	$1.1 \times 10^{-5} - 4.6 \times 10^{-5}$
			3	15	1.4×10^{-5}	1.7×10^{-5}	$7.0 \times 10^{-5} - 3.0 \times 10^{-5}$
			4	10	9.1×10^{-5}	5.6×10^{-5}	$2.3 \times 10^{-5} - 9.5 \times 10^{-5}$
			5	5	1.7×10^{-5}	3.8×10^{-5}	$1.4 \times 10^{-5} - 7.2 \times 10^{-5}$

M = male; F = female; BS = brood size; †Result first reported in original source; --- indicates not applicable or sample size too small to test.

in fish (Charnov *et al.* 1995), *Daphnia* (Chapter 3) and parasitoid wasps (Mayhew 1998; West *et al.* 2001) have found that variance in offspring size among broods decreases with increasing brood size. However, this decrease in variance has rarely followed closely the predictions of Charnov *et al.*'s invariant, and the decrease in variance was generally slower than predicted, as was the case here.

We know of three tests of Downhower and Charnov's invariant. Two are generally supportive of the invariant, in a fish (Downhower & Charnov 1998) and in *Daphnia* (Chapter 3). Mayhew (1998), using the data discussed here, found that variance in host weights increased with increasing brood size, suggesting that offspring were 'costlier' in larger broods. Here, the data for the most part did not fit Downhower and Charnov's invariant rule (figure 4.3; table 4.3). Those species that did not fit the invariant pattern had a positive correlation between brood size and host size at small host sizes, but some larger hosts harboured broods that were much larger than the average brood size for that host size.

Examination of where the invariants do not hold can prove useful in expanding our knowledge about parasitoid wasp reproduction, and tell us more generally about resource investment into offspring. Possible reasons why we did not find a strong quantitative fit to the invariant rules are discussed below.

First, Equation 4.1 assumes that the average amount of resources received by an offspring is constant over brood size. This assumption did not hold in this study, as body size was negatively correlated with brood size for half the species tested here (three of six). Under favourable conditions, it might pay females to increase number of offspring at the cost of offspring size (Hutchinson 1951; Green 1966) if host size is correlated with host abundance (or host finding rates) in the next generation. There is evidence that egg size is negatively correlated with clutch size in *Daphnia*, where mothers with the lowest food availability produce the largest offspring (Guinnee *et al.* 2004). However, in parasitic wasps, resources for offspring do not

come directly from the mother, but from the host. Why would a mother increase the number of offspring on one host when, if hosts are abundant, she can simply move on to the next host and lay more eggs there? One possibility is that the pattern reflects parent-offspring conflict – explicit theory would be useful to address this point (Godfray and Parker, 1991).

In this study, we do not take into account variables such as host instar number, or host size, when examining the brood size/wasp body size relationship, as we are looking for general patterns. It might be that we have missed something important by glossing over these points. However, there are many explanations why offspring size might vary with brood size, most involving trade-offs (see Mayhew & Glaizot 2001 and references therein). In other organisms (Westoby *et al.* 1992; Rees & Westoby 1997), and parasitoid wasps (Mesterton-Gibbons & Hardy 2004), complicated size-fitness relationships have been described, for example where fitness depends on an individual's size relative to competitors. In addition, for parasitoid wasps, it has been demonstrated theoretically that multiple optimal brood sizes might exist (Mesterton-Gibbons & Hardy 2004). It has also been demonstrated theoretically that variation in offspring size within an individual brood can be advantageous (Geritz 1995).

Second, while it has been shown that female wasps assess host size and adjust brood size accordingly (Godfray 1994; figure 4.3; table 4.3), their assessment might be imperfect. If host-size assessment is less accurate at larger host sizes, this would increase variability in wasp size at larger brood sizes. Alternatively, if there exists an asymmetric cost to producing too large a brood, the mother might favour the smaller brood size (Godfray & Ives 1988). This could increase offspring size-variability at all brood sizes, in effect 'washing out' Charnov *et al.*'s invariant. In addition, here brood size is defined as the number of young found on/in, or emerging from a host, therefore developmental mortality is not taken into account, and will likely affect body-size variance (although Mayhew 1998 performed similar analyses using only broods with no developmental mortality and found similar results as those reported

here: variance in wasp size decreased with increasing brood size but slower than predicted by theory).

Third, with the exception of *A. zwoelferi*, the species analysed here produce mixed sex broods. There might exist different levels of optimum investment in male and female offspring – the fact that females were bigger than males in five of six species here supports this. These differences have not been incorporated into theory, which assumes single sex broods, and further modelling is required to examine the extent to which the invariant predictions are expected to hold with mixed sex broods.

Fourth, while female wasps will attempt to avoid super-parasitism, multiple broods are sometimes laid on the same host; super-parasitism is more likely in data collected from the field. This might explain why *A. pallipes* and *M. stanleyi* both contained a few very large sized broods that were not on very large hosts (figure 4.3). It might also explain why, for some species, body size decreased with increasing brood size, as very large broods might in fact be multiple broods competing for the same host resources. Super-parasitism is not included in small brood size theory and would increase variance in body size between broods at larger brood sizes.

CHAPTER 5

WHY DO FOOD LIMITED MOTHERS PRODUCE LARGER OFFSPRING?: A THEORETICAL APPROACH

To be submitted as: M.A. Guinnee, A. Gardner, T.J. Little and S.A. West. Why do food limited mothers produce large offspring?: A theoretical approach, *The American Naturalist*

5.1 Abstract

We explore and expand upon a mathematical model first described by Charnov *et al.* (Charnov & Downhower 1995; Charnov *et al.* 1995) to investigate the relationship between offspring (or egg) size and clutch size. We determine that the negative relationship between egg size (or offspring size) and food availability (or clutch size) observed in *Daphnia* (a small freshwater crustacean) could result from either a minimum viable egg size, or selection for larger eggs at lower resource levels. Additionally, it has previously been shown that the range in egg size is predicted to decrease with clutch size in a predictable, invariant way. We explore how these predictions change with selection on egg size and minimum viable egg size.

5.2 Introduction

While the trade-off between offspring size and number is one of the most studied areas of evolutionary biology (e.g. Smith & Fretwell 1974; Roff 1992; Stearns 1992), we still do not have a complete understanding of why offspring (or egg) size variation exists within species. Recently, several theoretical models have addressed this problem and explicitly examined the consequences of small integer numbers for clutch size evolution (Ebert 1994; Charnov *et al.* 1995; Charnov & Downhower 1995; Downhower & Charnov 1998; West *et al.* 2001).

Charnov *et al.* (1995; Charnov & Downhower 1995) explored one such model and described an invariant rule describing how range in offspring (egg) size is related to clutch size (Equation 5.1; West *et al.* 2001).

$$\frac{I_{\max\_i} - I_{\min\_i}}{I_{\max\_j} - I_{\min\_j}} = \frac{C_j}{C_i} \qquad (5.1)$$

where I is offspring size, C is clutch size, $I_{max\,i}$ and $I_{min\,i}$ are the maximum and minimum size of offspring in clutches of C_i young, i and j are clutches of different sizes, and $i=j+1$. Invariants are unit-less, and independent of underlying trade-offs, such as the relationship between offspring size and fitness. This invariant has been tested in a fish (*Gambusia hubbsi*; Charnov & Downhower 1995), parasitoid wasps (Mayhew 1998; West *et al.* 2001; Chapter 4), and a *Daphnia* (Chapter 3). In general, these studies found that range in offspring (or egg) size decreased with increasing clutch size, but not necessarily at the rate predicted by the model. Here, we explore and expand upon Charnov *et al.*'s model. We look at the impact of a minimum viable egg size, or selection for larger eggs when resources are limited, on mean offspring (egg) size and the offspring (egg) size range invariant. We use data from *Daphnia* to test the predictions of this model.

5.3 The model

Dimensionless clutch size invariants

Charnov *et al.* (1995; Charnov & Downhower 1995) modelled the trade-off between offspring size and offspring number for small clutch sizes. Offspring survival is related to offspring size according to the expression:

$$S = S_m\left(1-\exp[-d(I-I_0)]\right), \qquad (5.2)$$

where $I > I_0$ is offspring size, I_0 is minimum viable offspring size, S_m is the asymptotic survival approached as $I \to \infty$, and the parameter d determines shape of the survival curve. The diminishing returns (Figure 5.1) from investment into a particular offspring means that, for a given clutch size (C), total offspring fitness is maximised by allocating total resources (R) equally within the clutch, i.e. each offspring is of size $I = R/C$. The fitness function to be maximised by natural selection is:

$$w \propto \left(R/I\right)\left(1-\exp[-d(I-I_0)]\right). \qquad (5.3)$$

Note that the value of the constant S_m is irrelevant, as it merely provides a constant rescaling of absolute fitness. The parameters d and I_0 have units of size^{-1} and units of size respectively, R is the total of offspring size, and hence has units of size, and the strategy I also has units of size.

We can re-arrange this fitness function so as to describe the maximisation problem in a dimensionless way:

$$w \propto \left(R/I\right)\left(1-\exp[-d\,I_0((I/I_0)-1)]\right). \qquad (5.4)$$

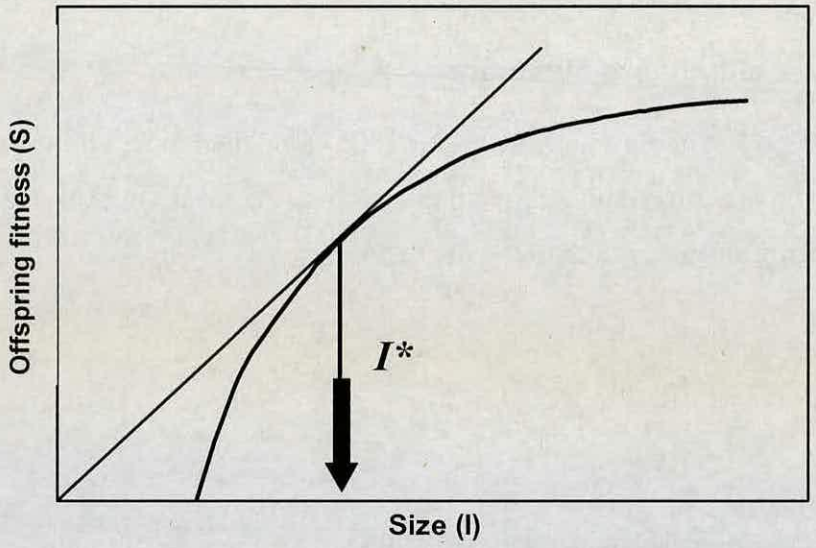

Figure 5.1: The curve relating offspring size to offspring fitness. Offspring fitness increases with parental investment per offspring (I), here measured as offspring size. The optimal offspring (egg) size (I^*) that maximises maternal fitness is defined as offspring (egg) size where the line through the origin is tangent to the curve.

The units of R and I cancel in the fraction R/I, as do those of d and I_0 in the product $d \times I_0$ and I and I_0 in the fraction I/I_0. Thus, the fitness function (equation 5.4) is made up of a set of dimensionless quantities. There are two immediate advantages of re-phrasing the maximisation problem in this way. Firstly, unit measures of offspring size are entirely arbitrary. A researcher measuring offspring size in grams will have different values of d and I_0 than a researcher who prefers to use ounces, although the pair should agree on the values of the dimensionless quantity $d \times I_0$. Secondly, it removes (unimportant) differences in scale. Any set of maximisation scenarios satisfying this model and sharing the same value of $d \times I_0$ are therefore equivalent, and hence will share the same values for R/I^* and I^*/I_0. The appropriate biology of a species with a minimum viable size of several milligrams can be understood as being equivalent to that of a species where minimum viable size is on the order of several kilograms, if they have similar $d \times I_0$.

For large clutches, the set of I strategies is approximately continuous, and optimal investment into offspring (I^*) is that which maximises marginal survival: the tangent to the curve at $I = I^*$ passes through the origin (figure 5.1). For smaller clutches, $R/I^* = C$ is constrained because C necessarily takes integer values. Since mothers will not in general have an amount of resources R which is an integer multiple of the 'optimum' I^*, they are constrained to produce 'non-optimal' clutches. Charnov *et al.* (1995; Charnov & Downhower 1995) gave a heuristic demonstration that the pattern of switch points – the (relativised) values of R where mothers switch from producing C offspring each of size R/C to producing $C+1$ offspring each of size $R/(C+1)$ – is approximately invariant to changes in the parameters and the basic model (Charnov & Downhower 1995; Charnov *et al.* 1995; West *et al.* 2001). Specifically, the minimum and maximum offspring sizes observed among the set of clutches of size C are expected to lie either side of (and be equidistant from) the optimum (I^*) that maximises fitness function (equation 5.4) when the integer clutch size condition is relaxed. The difference between this maximum and minimum for clutch size C is expected to be a fraction $(C)/C+1$ of that for clutch size C (equation 5.1). While Charnov *et al.* used the equations described above, they found that this relationship (equation 5.1) holds for a variety of trade-off fitness functions (Charnov & Downhower 1995). The minimum and maximum offspring sizes are expected to converge on the 'optimum' I^* as clutch size increases. We will investigate how this pattern changes with regards to: (1) variation in the dimensionless parameter $d \times I_0$; and (2) $d \times I_0$ dependent on maternal resources, R.

Variation in $d \times I_0$

As described above, a system can be characterized according to one dimensionless parameter, $d \times I_0$. Sets of systems with the same value of $d \times I_0$ are equivalent in the sense that the relative maternal resource value ($R/I_0 = (R/I^*) \times (I^*/I_0)$) at which a mother switches from producing C offspring of size R/C to $C+1$ of size $R/(C+1)$ is invariant. We will now investigate how variation in $d \times I_0$ translates into variation in

this pattern. An increase in $d \times I_0$ can be thought of as increasing d while holding I_0 fixed. Increasing d increases the steepness of the survival slope. At higher values of d, there is a stronger survival advantage for being slightly larger for offspring close to the minimum viable size, but very little advantage of increasing size when offspring size takes larger values (figure 5.2). In figure 5.3 we see that variation in $d \times I_0$ translates into variation in the relative size ranges for clutches of one or two offspring, but the invariant holds rather well for larger clutch sizes. In the limit, as $d \times I_0$ takes large values, the invariant holds perfectly. Some examples of the switch point pattern are given for $d \times I_0 = 0.1$, 1.0 and 10 (figure 5.4).

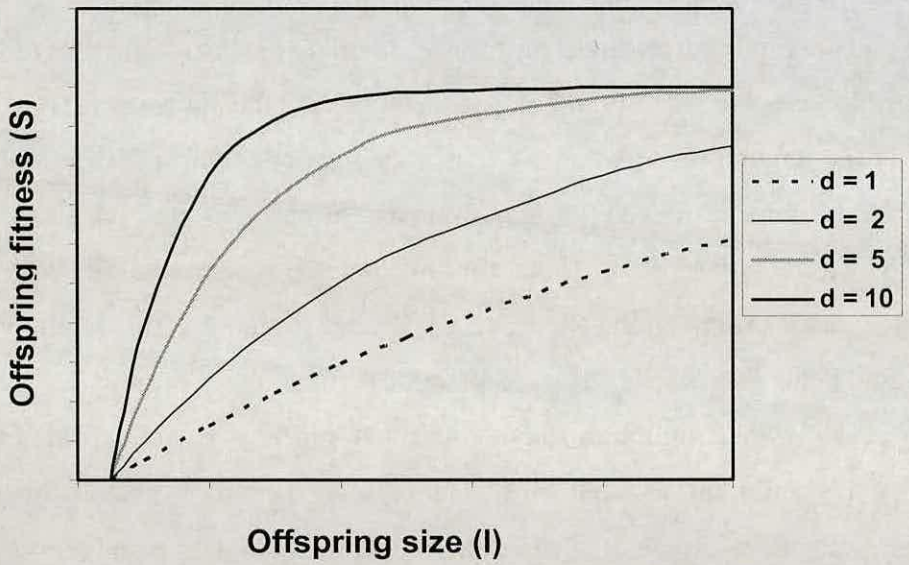

Figure 5.2: The steepness of the fitness slope increases at higher values of *d*. At high values of *d*, there is a strong advantage to being slightly larger for offspring close to the minimum viable size, but very little advantage of increasing size when offspring size takes larger values. Lower values of *d* predict a larger optimal offspring size (*l**).

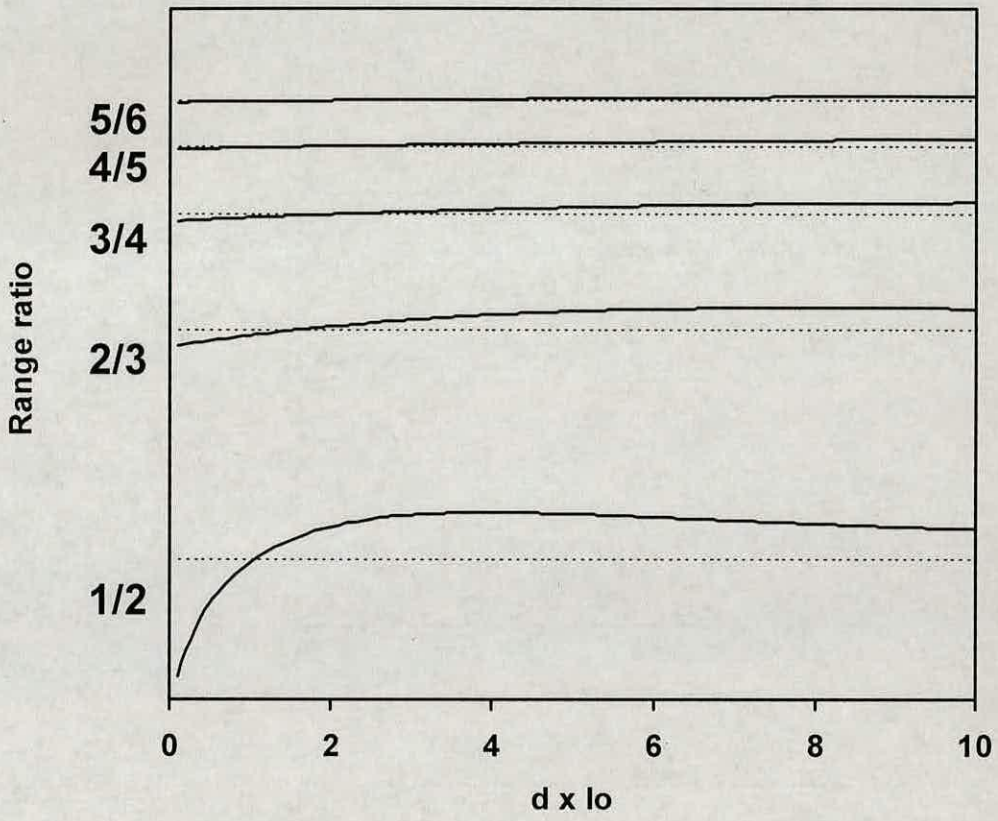

Figure 5.3: Invariant range ratios predicted by equation 5.1 (dotted lines) and those predicted for different $d \times l_0$ values (solid lines).

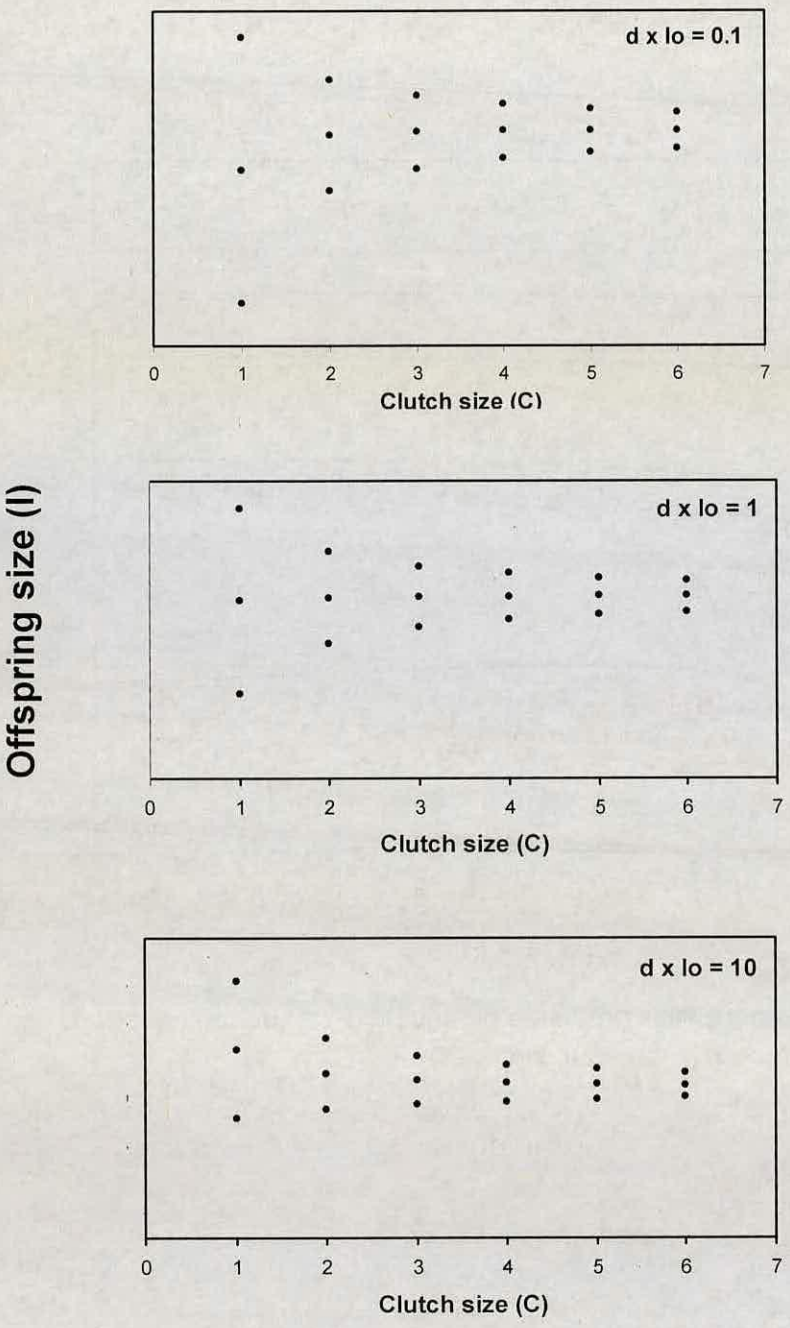

Figure 5.4: Switch point patterns for varying values of $d \times l_0$. The upper point refers to the largest offspring size and the lower point to the smallest ffspring size predicted for that clutch size. The distance between the points represents the total range of offspring sizes expected for that clutch.

Very high values of $d \times I_0$

In the previous sections we discovered that the Charnov approximate invariant in offspring allocation strategy holds perfectly in the limit of $d \times I_0 \to \infty$, i.e. as offspring survival approaches the step function:

$$S = \begin{cases} 0 & I < I_0 \\ \quad if & \\ S_m & I > I_0 \end{cases} . \qquad (5.5)$$

In this limit, there is vanishingly little advantage in allocating resources to individual offspring beyond what is required to make them viable (figure 5.5). Hence, resources should be allocated to a new offspring as soon as enough are available to make it viable, and I^* approaches Io. This gives rise to the pattern in figure 5.6.

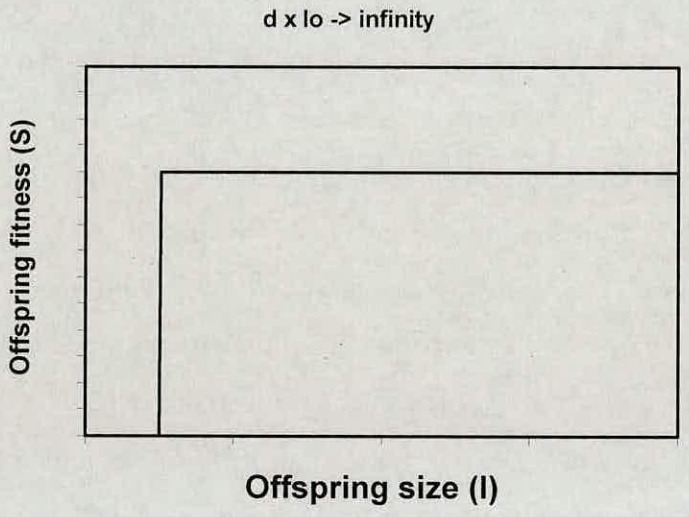

Figure 5.5: Fitness curve extreme values of *d*. As *d* approaches infinity, the steepness of the fitness slope increases until there is no advantage in allocating resources to individual offspring beyond what is required to make them viable. Hence, optimal offspring size is equal to minimum viable offspring size ($I^* = Io$).

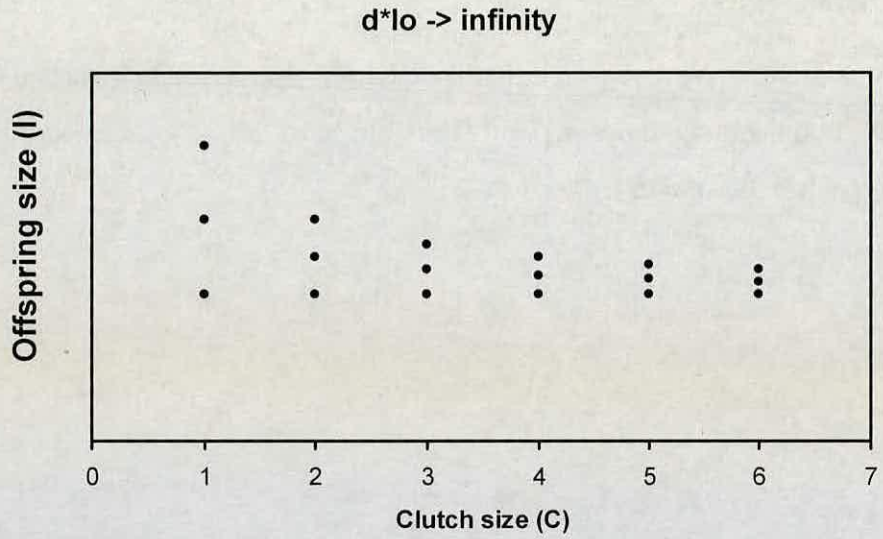

Figure 5.6: Switch point pattern when $d \times I_0$ equals infinity. There is no advantage in allocating resources to individual offspring beyond what is required to make them viable ($l^* = lo$). The upper point refers to the largest offspring size and the lower point to the smallest offspring volume predicted for that clutch size. The distance between the points represents the total range of egg sizes expected for that clutch.

$d \times I_0$ as a function of R

We now investigate the effects of variation in $d \times I_0$ within a population; namely, where $d \times I_0$ is a function of relative maternal resources, R/I_0. A positive correlation between $d \times I_0$ and her relative resources (R/I_0) might be expected if the fitness benefit of being a large offspring is greater in low-food environments (Hutchinson 1951; Green 1966; Goulden *et al.* 1987; Gliwicz & Guisande 1992), and if maternal environment is indicative of offspring environment. For simplicity, we assume a proportional relationship: $d \times I_0 = \lambda R/I_0$. The effect of this model change on Charnov *et al.*'s invariant is illustrated in figure 5.7, for a range of λ. Again, implementing this change in the model significantly disturbs the invariant relationship only for small clutch sizes. For all clutch sizes, the effect is reduced as λ increases (figure 5.8) – as expected, since increased λ translates into increased $d \times I_0$, which was investigated in figure 5.4. Some examples of the switch point pattern are given for λ = 0.1, 1.0 and 10 (figure 5.9).

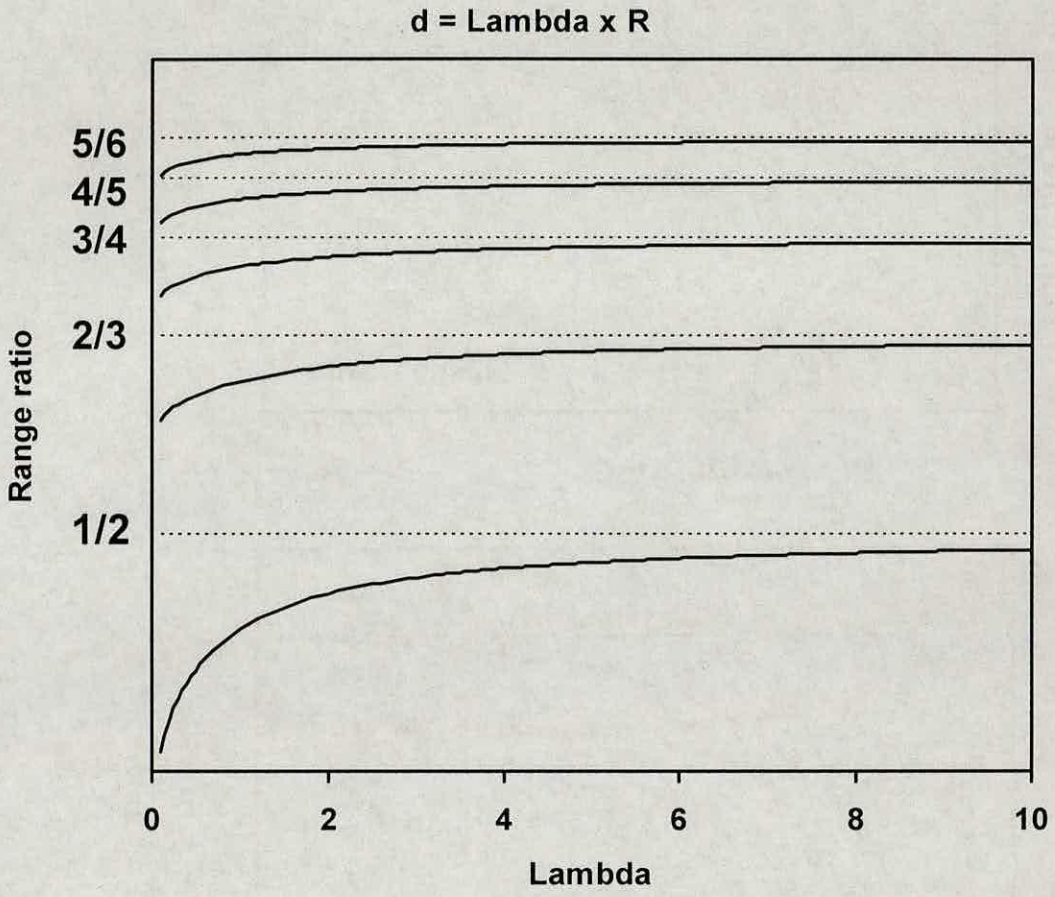

Figure 5.7: Invariant range ratios predicted by equation 5.1 (dotted lines) and those predicted for different λ values (solid lines) where $\lambda = d^*R$.

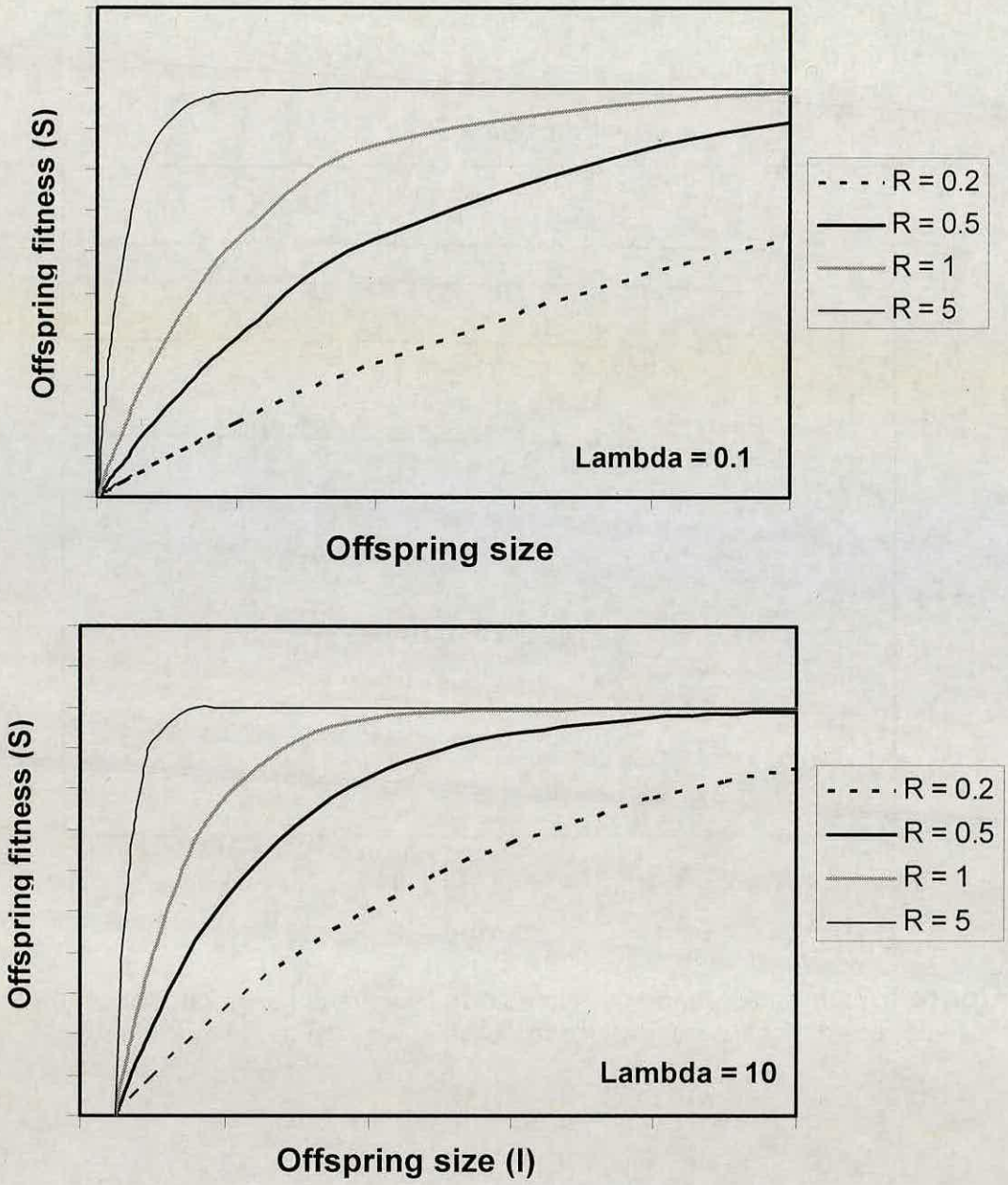

Figure 5.8: The steepness of the fitness curve varies with resources (R) and λ. The more resources (*R*) available to an individual, the faster the function relating offspring size to offspring fitness approaches an asymptote, and the smaller the optimum offspring size (*l**). You will notice that this figure looks very much like that of figure 5.2; that is because changing *R* in this case is similar to changing *d* in figure 5.2: both change the steepness of the fitness function. However, for the case of figure 5.2, there is one value of *d*, regardless of environment. In figure 5.4, *d* is modified by *R*, meaning that the steepness of the curve is dependent upon environment.

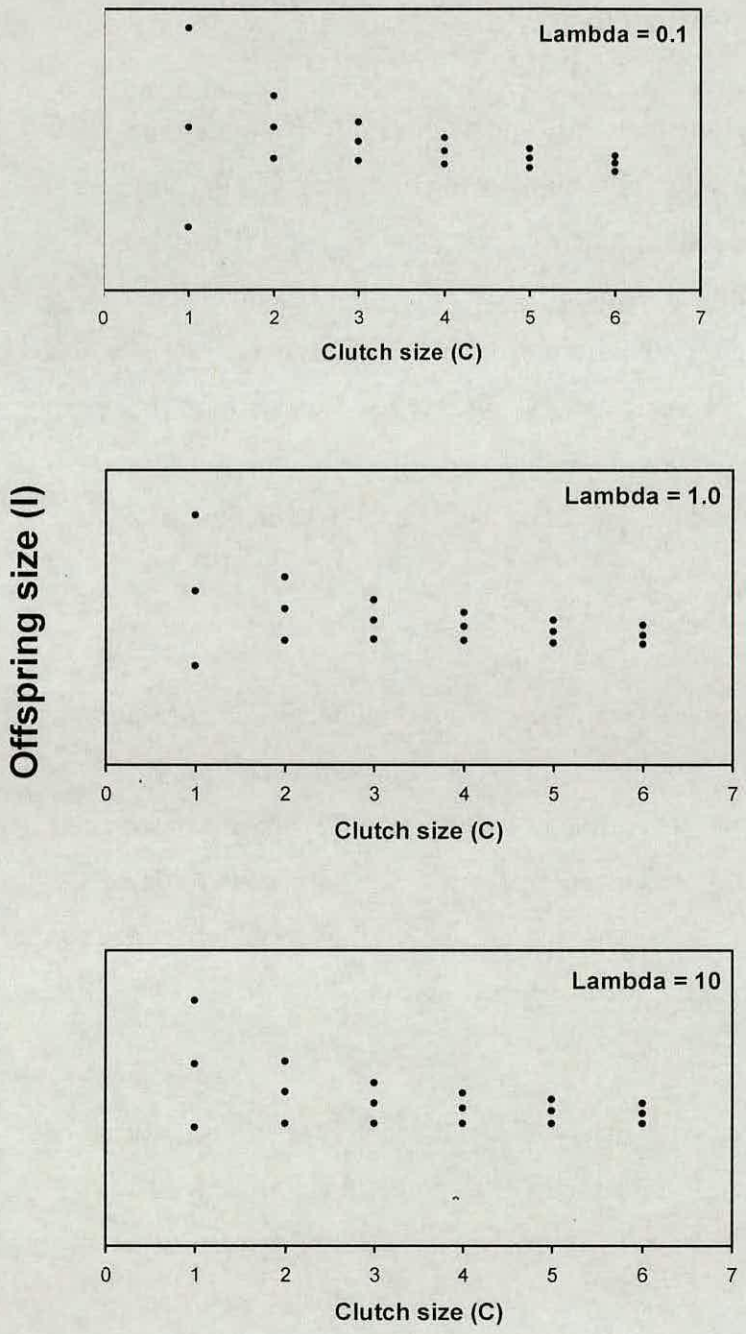

Figure 5.9: Switch point patterns for varying values of λ. The upper point refers to the largest offspring size and the lower point to the smallest offspring size predicted for that clutch size. The distance between the points represents the total range of offspring sizes expected for that clutch.

5.4 Testing the model using *Daphnia*

Daphnia are an ideal organism for empirically and theoretically examining the relationship between resource availability and per-offspring resource allocation as: (1) much is known about the relationship between food and egg size (Chapter 3; table 5.1); (2) we can experimentally manipulate the amount of resources a female has for reproduction by varying the amount of available food; (3) they are easily kept in large numbers in the lab, so we can explore a large range of resource levels; (4) they are clonal, which allows us to compare genetically identical individuals; and (5) *Daphnia* with more resources produce larger clutches (Chapter 3), so clutch size is a measure of resource availability.

Here, we assume that egg size and offspring size are accurate measures of resource allocation in *Daphnia*. Egg size correlates with eventual neonate size (Lampert 1993; Ebert 1994), and offspring size correlates with fitness (Tessier & Consolatti 1989; Gliwicz & Guisande 1992), suggesting that larger eggs/larger offspring have more resources. Our aim here is to explore the hypotheses, models and evidence for the influence of food availability on egg and neonate size in *Daphnia*.

Most studies examining the phenomenon have found a negative relationship between egg (or offspring) size and food (or clutch size) in *Daphnia* (table 5.1). We have found no data in the literature on range in offspring (egg) size and food availability and/or clutch size. In Chapter 3, we found a statistically significant negative relationship between food and egg size (figure 3.3). That was the first experiment to explore the relationship between egg size and food treatment using a very large number of food treatments, and is thus perhaps the first experiment with sufficient power to test the relationship between egg size and food availability. The increments

Table 5.1: Studies examining the relationship between resources available to a mother and change in mean egg size in *Daphnia*.

Source	Species	Measure of resource investment	Change in size with increase in food (or clutch size, if stated)	Number of treatments
Boersma 1995	*D. galeata*	Egg carbon content	Dome-shaped	4
Gliwicz & Guisande 1992	*D. hyalina*	Neonate length	Decrease	3
Enserink *et al.* 1990	*D. magna*	Neonate length	Decrease	4
Glazier 1992	*D. magna*	Egg mass	Decrease or no relationship (2 clones)	2
Ebert 1993	*D. magna*	Neonate length	Decrease	13
Enserink *et al.* 1993	*D. magna*	Neonate length	Decrease	3
Trubetskova & Lampert 1995	*D. magna*	Egg length and egg mass	Decrease	2
Boersma 1997	*D. magna*	Neonate mass	Decrease	2
Taylor 1985	*D. pulex*	Egg mass	Decrease	2
Lynch 1989	*D. pulex*	Egg mass	Increase	9
Tessier & Consolatti 1991	*D. pulex*	Neonate length	Increase	3
LaMontagne & McCauley 2001	*D. pulex*	Neonate length	Increase	2
Taylor 1985	*D. pulicara*	Egg mass	No change	2
Tessier & Consolatti 1991	*D. pulicaria*	Neonate length	Dome-shaped	3
Gliwicz & Guisande 1992	*D. pulicaria*	Neonate mass	Decrease	3

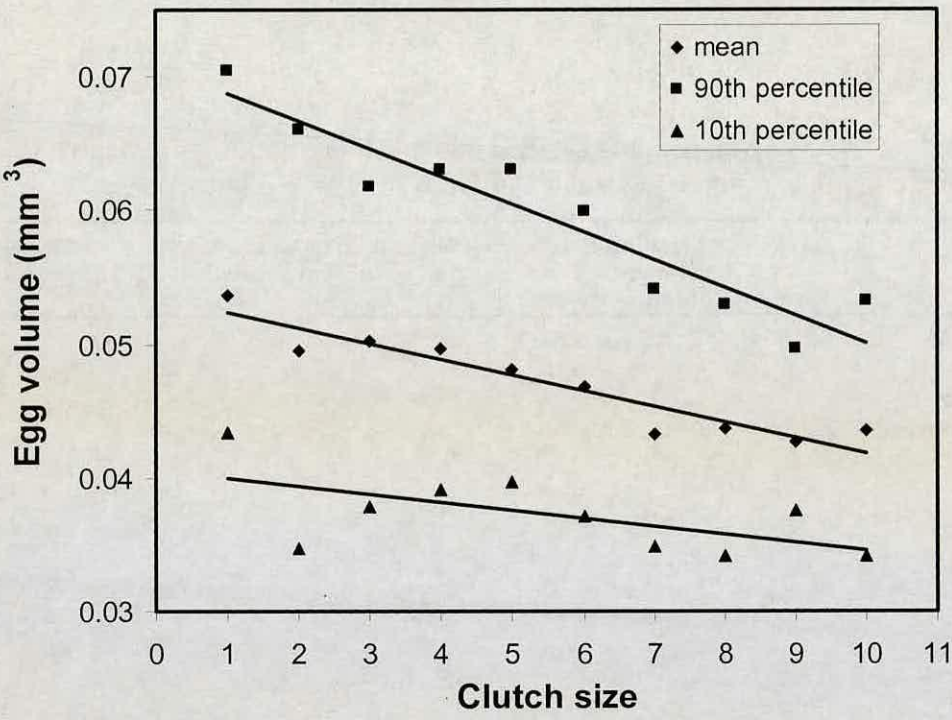

Figure 5.10: Mean (diamonds), 90th percentile (triangles) and 10th percentile (squares) for egg volume for clutches of size 1-10. Linear trend line: Mean: y = -0.0012x + 0.054, r2 = 0.89; 90th percentile: y = -0.0021x + 0.071, r2 = 0.89; 10th percentile: y = -0.0006x + 0.041, r2 = 0.37. Figure is reproduced from Chapter 3.

in food level were very small and the *Daphnia* failed to reproduce at the lowest food levels. We also found a significant decrease in variance in egg size (between clutches) as clutch size increased (figure 5.10). However, the decrease did not closely follow the predictions of Equation 5.1.

Our *Daphnia* data (figure 5.10) look very similar to figure 5.6 and figure 5.9 (where $\lambda = 10$). Interestingly, Charnov *et al.* (1995) found a pattern similar to figure 5.10 in a fish (*Gambusia hubbsi*). The similar patterns in figures 5.6 and 5.9 come about for very different reasons. In figure 5.6, where $d \times I_0$ is high, there is no advantage in allocating resources to individual offspring beyond what is required to make them viable ($I^* = Io$), and optimal offspring size does not vary with resources (R). In figure 5.9, where $\lambda = 10$, the steepness of the fitness function is highly dependent on resources (R), and the optimum offspring (egg) size (I^*) varies greatly with resource

availability. As resources increase, clutch size also increases, and optimal egg size decreases.

How could we test whether differences in mean egg size at different clutch sizes in *Daphnia* are due to either of these behaviours? If this relationship is indeed caused by a minimum viable egg size it suggests that $d \times I_0$ is very high indeed, and that the optimum egg size is very similar to the minimum viable egg size. To further test this we would need to know the shape of the offspring size / offspring fitness curve, something that has been worked out for few species (e.g. Sehgal & Toor 1991; West *et al.* 1996; Bennett & Hoffmann 1998; Einum & Fleming 2000; Bezemer & Mills 2003). The best available data in *Daphnia* is provided by Bell (1983), who found a steep function relating egg size and likelihood of hatching in *Daphnia*, and eggs below a certain size did not hatch at all (figure 5.11). Because Bell used a different species (*Daphnia pulex*) than we used to generate figure 5.10, egg volumes are not directly comparable to our results, but, it is likely that general patterns would be similar across *Daphnia* species. Generally for *Daphnia*, large eggs hatch into large offspring (Chapter 6; Lampert 1993; Ebert 1994), and larger offspring have increased juvenile survival under starvation conditions (Tessier & Consolatti 1989; Gliwicz & Guisande 1992). This suggests that the egg size / offspring fitness curve for *Daphnia* is actually steeper than that found by Bell (1983; figure 5.11), and while figure 5.5 is quite extreme, in reality, the egg size / offspring fitness curve in *Daphnia* might not be far off. This means that the optimum egg size might be very similar to the minimum egg size ($I^* \approx Io$). But is such a pattern biologically feasible? If the optimum egg size is similar to the minimum viable egg size, *Daphnia* have little room for error – if they produce eggs that are the slightest bit smaller than the optimum the fitness of the clutch will be zero.

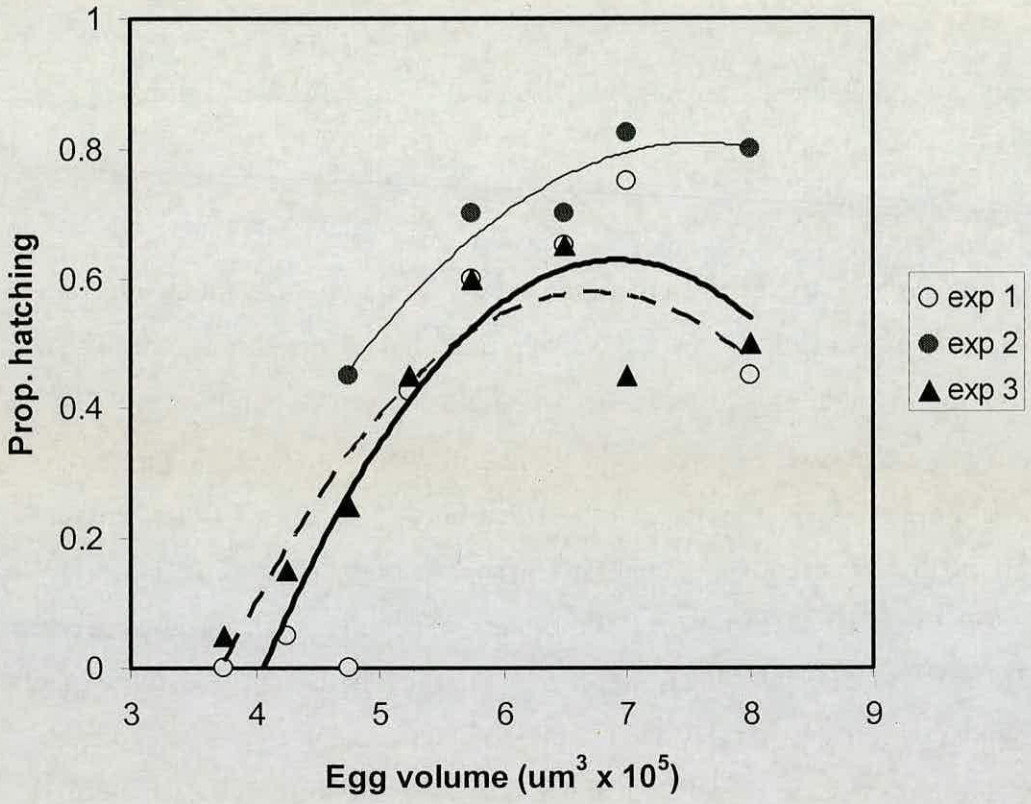

Figure 5.11: Likelihood of hatching increases with egg volume. Figure is reproduced from Bell (1983), and shows data from 3 separate experiments. Polynomial (incorporating a squared term) lines of best fit: Experiment 1: $y = -0.077x2 + 1.06x - 3.05$, $r^2 = 0.81$; Experiment 2: $y = -0.044\ x2 + 0.66x - 1.71$, $r^2 = 0.93$; Experiment 3: $y = -0.065\ x2 + 0.87x - 2.34$, $r^2 = 0.88$. See original publication for details on materials and methods.

Alternatively, we have demonstrated that the pattern we observed empirically in *Daphnia* could theoretically result from selection for smaller eggs when resources are abundant (figure 5.9, $\lambda = 10$). *Daphnia* exhibit extreme plasticity in response to their environment (see General Introduction for a review) so it would not be surprising if they adjusted egg size in line with food availability, and this has been suggested by multiple researchers (e.g. Green 1966; Goulden *et al.* 1987; Gliwicz & Guisande 1992). In addition, *Daphnia* have a short generation time, therefore it is reasonable to expect that maternal food availability is indicative of offspring food availability (Boersma 1997). Food availability in the wild is likely to be highly variable (Gliwicz & Guisande 1992), so phenotypic plasticity is likely to have evolved if linking egg size to maternal resource availability is indeed beneficial to maternal fitness.

5.5 Conclusions

Propagule size is one of the most active areas of life-history research as it can have large fitness implications for both the offspring and its parents (Roff 1992; Stearns 1992; Bernardo 1996b; Mousseau & Fox 1998). *Daphnia* generally produce larger offspring (or eggs) when food is limited (table 5.1; Chapter 3; figure 5.10). It is often suggested that mothers produce larger offspring when food-limited because the benefits of being a large offspring are greater in food-limited environments (Hutchinson 1951; Green 1966; Goulden *et al.* 1987; Gliwicz & Guisande 1992). Our study has shown that this might be the case, but not necessarily. We have built upon a mathematical model first described by Charnov *et al.* (1995; Charnov & Downhower 1995) to explore theoretically the effect of a minimum egg size and selection for smaller eggs at higher resource levels. We found that a decreasing mean egg size with increasing clutch size could result from selection for smaller eggs when resources are abundant. For this to fit our *Daphnia* data (figure 5.10), egg size must be highly dependent on maternal resources. Alternatively, we have also demonstrated that this pattern could be the by-product of a minimum viable egg size that is similar to the optimum egg size. Discriminating between the above two hypotheses will require further empirical data. Specifically, it would be extremely useful to have a size/fitness curve for *Daphnia*. Fortunately, *Daphnia* are one of the few species in which a study describing the size/fitness curve in multiple environments is feasible. In addition, further tests in other species should be done to explore the generality of the model tested here.

CHAPTER 6

DO BIGGER OFFSPRING PRODUCED BY RESOURCE-LIMITED *DAPHNIA* HAVE HIGHER FITNESS?

To be submitted as: M.A. Guinnee, A.E. Howard, S.A. West and T.J. Little. Do bigger offspring produced by resource-limited *Daphnia* have higher fitness?

6.1 Abstract

Food-limited mothers often produce fewer, but larger, offspring. We tested whether larger offspring produced by food-limited *Daphnia magna* have higher fitness. We placed individuals into high or low food. As expected, the *Daphnia* in the low food treatment produced larger eggs than those in the high food. We then placed the offspring hatching from those eggs into the same food treatments in a fully factorial design, and measured their age at maturity, growth, reproduction and the size of their offspring for their first three clutches. While offspring from low food mothers were larger at birth, they matured later, and produced fewer offspring which were smaller (when compared to individuals currently at the same food level). These results suggest that reproductive life-history traits are not simply a function of resources, and that offspring from food-limited mothers are programmed to be more cautious, investing resources into survival rather than reproduction. We also found that the amount of resources a *Daphnia* provisioned to its offspring was dependent both on its current food treatment and that of its mother, with the largest offspring produced by individuals at low food conditions whose mothers had been at high food. Our findings suggest that current environment, maternal environment and grand-maternal environment could all influence an organism's key fitness traits.

6.2 Introduction

Larger offspring are generally more fit (see Roff 1992; Mousseau 1998 and references therein), but mothers which produce larger offspring must produce fewer offspring, because of a trade-off between offspring size and offspring number (Smith & Fretwell 1974). It has been hypothesized that the fitness benefit of being a large offspring should be greater in food-limited environments in *Daphnia* (Hutchinson 1951; Green 1966; Goulden *et al.* 1987; Gliwicz & Guisande 1992). Consequently, *Daphnia* in poor environments should produce larger offspring if: (1) maternal environment is indicative of offspring environment; and (2) the environment is variable enough that plasticity in offspring size will have evolved.

Daphnia have a short generation time, therefore it is reasonable to expect that maternal food availability is indicative of offspring food availability (Boersma 1997). Food availability in the wild is likely to be highly variable (Gliwicz & Guisande 1992), and *Daphnia* have extremely plastic life-history characteristics (see General Introduction) and consequently are likely to respond to food availability cues. In addition, maternal environment has previously been demonstrated to influence growth and reproduction (Lynch & Ennis 1983), survival (Lynch & Ennis 1983; Gliwicz & Guisande 1992; Cleuvers *et al.* 1997), resting egg production (LaMontagne & McCauley 2001; Alekseev & Lampert 2001), morphology (Agrawal *et al.* 1999), and susceptibility to parasitism (Little *et al.* 2003) in *Daphnia*. In various species of *Daphnia* (table 5.1), and specifically for the genotype we use here (Chapter 3), food-limited mothers produce larger eggs, supporting the hypothesis that food-limited mothers provision individual offspring with more resources. It has been demonstrated previously that larger offspring, or those offspring produced by food-limited mothers, have increased survival under low-food or starvation conditions (Lynch & Ennis 1983; Tessier & Consolatti 1989; Gliwicz & Guisande 1992). But little is known about the effects of maternal environment on reproduction (but see Lynch & Ennis 1983; LaMontagne & McCauley 2001).

In Chapter 3, we demonstrated that a genotype of *Daphnia magna* produces larger eggs in food-limited environments. Here, we place that same genotype into two different food environments (low and high). Food-stressed *Daphnia* produced larger eggs than the *Daphnia* with abundant food, as expected. We placed the offspring hatching from those eggs into the same two food environments (in a fully factorial design) and recorded fitness measures (growth, age at maturity, clutch size, offspring size). Our hypotheses are: (1) (larger) offspring produced by mothers in a food-limited environment will be fitter; (2) the fitness advantage of being born to a mother in a food-limited environment (and therefore being larger) is greater when the current food environment is food-limited.

6.3 Materials and Methods

Experiment 1: reproduction

Study organism:

We used a clone of *Daphnia magna* that had originated in the Gaarzerfeld pond, Northern Germany. *Daphnia magna* from this pond have been the focus of numerous studies of parasitism and life-history evolution (e.g. Little & Ebert 2001; Little *et al.* 2002). Throughout, *Daphnia* were fed on chemostat-grown culture of the green algae *Scenedesmus* sp. and kept in incubators (20 C, 14:10 light/dark cycle). We raised three generations of *Daphnia* in jars containing 200mL *Daphnia* media to equilibrate the conditions of the *Daphnia*. The first two generations had 5 *Daphnia* per jar with 1.5×10^7 algal cells per day per jar (for 72 jars x 5 *Daphnia*/jar = 360 *Daphnia*). The third generation was one *Daphnia* per jar, fed 1.2×10^6 cells/*Daphnia*/day, for 120 *Daphnia*. For each new generation, we combined all neonates produced over a 24 hour period (first clutch neonates were not used) and randomly allocated neonates to jars. We changed *Daphnia* media every-other day. *Daphnia* media was a modified version of the Aachener Daphnien Medium described by Klüttgen *et al* (1994).

Methods:

When the third generation produced their third clutch of offspring, one offspring from each *Daphnia* was placed into a separate jar containing 200mL *Daphnia* media. Each individual was randomly assigned to one of two treatments: high (H, 32 x 10^5 cells/*Daphnia*/day) or low (L, 4 x 10^5 cells/*Daphnia*/day). We refer to these *Daphnia* as the 'maternal generation'.

When they produced their second clutch of eggs, the maternal *Daphnia* (still containing eggs; ≤14 hours after egg production) were photographed using a camera attached to a dissecting microscope. We also photographed 3 eggs from each maternal *Daphnia* through the carapace. All eggs were in stage 1 of development as described by Threlkeld (1979).

When the maternal generation produced their second clutch, 2 offspring (≤ 14 hrs old) from each *Daphnia* were photographed using a camera attached to a dissecting microscope, and placed (randomly) into a treatment: H or L (treatments were identical to the maternal generation). We refer to these *Daphnia* as the 'current generation'. Where a *Daphnia* in the maternal generation produced only one offspring, that offspring was randomly assigned to a treatment. Figure 6.1 outlines the experimental design.

We checked the current generation *Daphnia* twice per day, morning and evening, and recorded deaths. We photographed the current generation *Daphnia* every-other day, and each time they produced a clutch of eggs. All offspring produced by the current generation were counted and sexed, and three offspring (randomly chosen) were photographed (females only; ≤ 14 hrs old). After a *Daphnia* produced three clutches, she was removed from the experiment. *Daphnia* jars were in trays, 12 jars to a tray. We randomised the location of the *Daphnia* within trays and the location

of the trays within the incubator. We systematically rotated the jars within the trays, and the trays within the incubator each day. Photographs were later used to measure body length (top of head to base of tail spine) and egg length (length of longest axis).

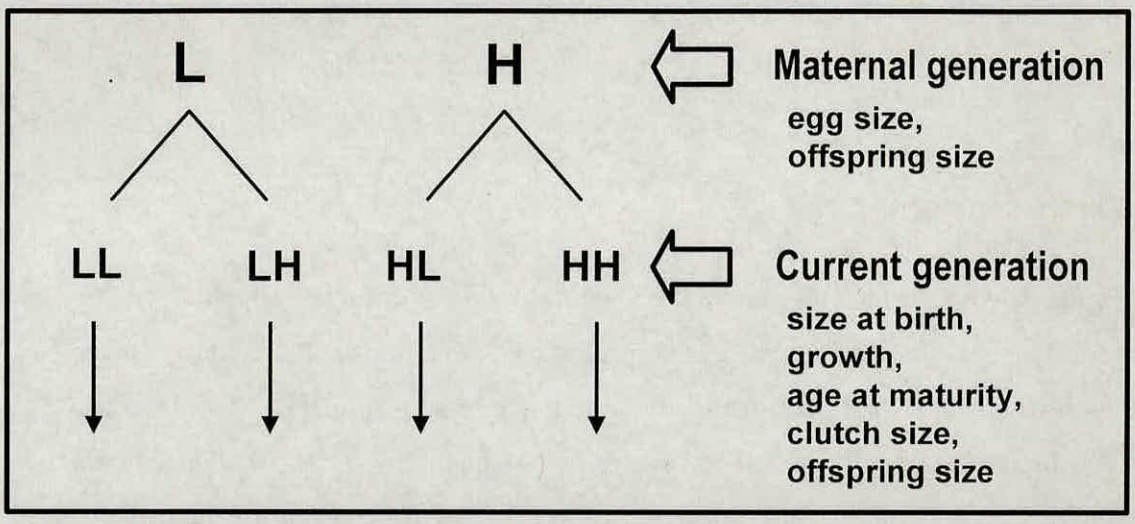

FIGURE 6.1: Experimental design. Food treatments were low (L, 4 x 10^5 cells/*Daphnia*/day) and high (H, 32 x 10^5 cells/*Daphnia*/day). Sample sizes for the maternal generations were L = 51, and H = 33. When a *Daphnia* in the maternal generation produced her second clutch, 2 of her offspring were randomly chosen and 1 was placed into each of two treatments, H or L, which were identical to the maternal treatments. Age and size at maturity, growth and reproduction were monitored for the current generation.

Statistical analyses:

We used the SAS System: Release 8.0 (SAS Institute) for all data analyses. All linear regression analyses and mixed effects models were done using the proc mixed command in SAS. All reported *P*-values are for two-tailed tests with a critical *P*-value of 0.05. Egg length and offspring length were averaged over mother to avoid pseudo-replication (Hurlbert 1984). Non-significant terms ($P>0.05$) were eliminated from models using backwards elimination (Crawley 1993).

Maternal generation

To confirm that egg size was dependent on maternal food availability, we tested for a relationship between the length of eggs produced by the maternal generation and food treatment using linear regression with the equation: egg length = food treatment. We determined whether offspring size was correlated with egg size using a Spearman's rank test to compare egg length to offspring length.

Current generation

We tested whether the current and the maternal food environment affected age at maturity (measured as the day eggs were first observed) and size at maturity (body length when eggs were first observed) using mixed effects models. Age at maturity was square-root transformed to correct for heterogeneity of variances. The equations were: response variable (for current generation *Daphnia*) = current treatment + maternal treatment + current x maternal treatment. Maternal *Daphnia* was included as a random factor.

We tested how the current and the maternal food environment affect how a mother provisions resources to her offspring by examining how the food treatments related to the size of the offspring produced by the current generation using a mixed effects model. The equation was: size of offspring (produced by current generation) = current treatment + maternal treatment + current x maternal treatment. Included as random factors were: maternal *Daphnia*, current *Daphnia*(maternal *Daphnia*), and clutch number(current *Daphnia*).

We tested whether the current and the maternal food environment affected growth in the current generation by testing for a difference in body length on each of days 2, 4, 6 and 8 (post-hatching) using mixed effects models. Body length was square-root transformed to correct for heterogeneity of variances. We used the equation: length (of current generation *Daphnia*) = current treatment + maternal treatment + current x

maternal treatment. Maternal *Daphnia* was included as a random factor. Each day was tested separately.

To look for a trade-off between clutch size and offspring size, we used a mixed effects model with the equation: birth length (of offspring produced by current generation *Daphnia*) = clutch size + maternal treatment + clutch size x maternal treatment. Clutch number(current *Daphnia*) was included as a random factor. This analysis was carried out separately for each current treatment.

Experiment 2: mortality

Study organism:

Study organism and continuation of lines were identical to Experiment 1 (above).

Methods:

Daphnia neonates (≤12hrs old) were collected and randomly assigned to high or low treatment groups in the same manner as for Experiment 1 (above). When the *Daphnia* produced a clutch, one randomly chosen offspring was placed in well of a 6-well, multiwell plate (Corning Incorporated, flat bottomed wells, approximately 3.5cm diameter) containing 6mL *Daphnia* media. Placement of the *Daphnia* in the wells, and the wells within the incubator, was randomised. *Daphnia* were examined once per day, and lifespan recorded in days.

Statistical analyses:

Because data were not normally distributed, a Wilcoxon two-sample test was carried out using the SAS System: Release 8.0 (SAS Institute).

6.4 Results

Experiment 1: reproduction

A total of 84 maternal generation and 138 current generation *Daphnia* were included in the experiment. A total of 151 eggs were measured, and 1833 neonates (produced by the current generation) were recorded, of which 685 were measured. There was little mortality during the experiment (deaths in the current generation: 0 LL, 0 LH, 1 HL, 1HH), and all surviving *Daphnia* reproduced.

Maternal generation:

Mothers in the low food treatment produced larger eggs than mothers in the high food treatment (mean ± sd: High: 0.36 ± 0.022mm, Low: 0.39 ± 0.033mm; $F_{1,70}$=12.1, P=0.0009). Egg length was positively correlated with neonate length, meaning that big eggs hatched into big offspring (n=72, r=0.40, P=0.0006; figure 6.2).

Current generation:

High current and maternal food treatment were associated with early maturation in the current generation *Daphnia* (current treatment: $F_{1,50}$=650.9, P<0.0001, maternal treatment $F_{1,50}$=33.3, P<0.0001). The current x maternal food treatment interaction was not significant ($F_{1,49}$=1.8, P=0.35; figure 6.3). *Daphnia* were larger at maturation when current treatment = high, and when maternal treatment = low (current treatment: $F_{1,47}$=175.1, P<0.0001; maternal treatment: $F_{1,47}$=12.5, P=0.0009). The current x maternal treatment interaction was not significant ($F_{1,46}$=0.03, P=0.85; figure 6.3). Because they reproduced later (figure 6.3), at any time in the experiment, *Daphnia* from low food mothers had produced fewer offspring than *Daphnia* from high food mothers (as compared to *Daphnia* in the same current food treatment; figure 6.4).

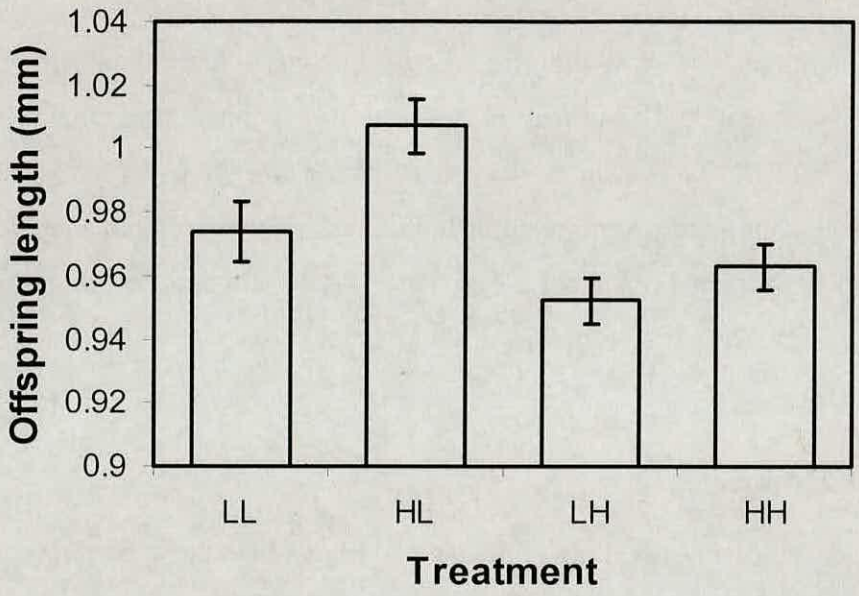

FIGURE 6.5: The mean offspring length (averaged over clutch) for each treatment. *Daphnia* in the current treatment = H produced smaller offspring than current treatment = L. *Daphnia* whose maternal treatment = H produced larger offspring than maternal treatment = L. Data are LSMeans, Error bars are ±s.e.

Both current and maternal food treatments affected how a mother provisioned resources to her offspring, as offspring produced by the current generation were smaller when current treatment = high, but larger when maternal treatment = high (current treatment: $F_{1,60}$=17.2, P<0.0001, maternal treatment $F_{1,60}$=6.1, P=0.017, current x maternal food treatment $F_{1,60}$=1.9, P=0.17; figure 6.5).

On day 2, *Daphnia* (current generation) from low food mothers were larger, while current treatment did not affect size (current treatment: $F_{1,13}$=1.0, P=0.33; maternal treatment: $F_{1,14}$=7.6, P=0.015). After day 2 (on days 4, 6 and 8), *Daphnia* at higher current food treatments were larger. While *Daphnia* who were produced by low-food mothers were generally smaller on days 4, 6 and 8 (when comparing within current treatment), maternal food treatment was not statistically significant (P>0.05 for maternal treatment and current x maternal treatment for all of Days 4, 6 and 8. P<0.0001 for current treatment for all of Days 4, 6 and 8; figure 6.6).

When current treatment = L, offspring from large clutches were smaller (clutch size: $F_{1,50}$=8.3, P=0.006; maternal treatment: $F_{1,49}$=2.4, P=0.13; clutch size x maternal treatment: $F_{1,48}$=0.1, P=0.83). Clutch size and offspring size were positively related for the HH group, but there was no relationship in the LH group (clutch size: $F_{1,94}$=1.0, P=0.32; maternal treatment: $F_{1,94}$=4.3, P=0.040; clutch size x maternal treatment: $F_{1,94}$=6.5, P=0.012; figure 6.7).

Experiment 2: mortality

Offspring produced by low-food mothers survived longer than those produced by high-food mothers for clutches 1, 2 and 3, although the difference was only statistically significant for clutch 3 (clutch 1: U_s=1455.6, P=0.75; clutch 2: U_s=1208.0, P=0.83; Clutch 3: U_s=1416.0, P=0.001; figure 6.8).

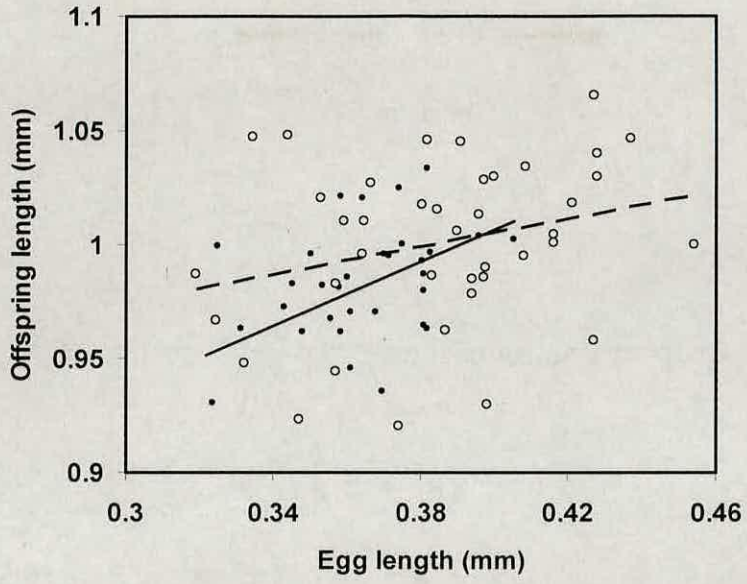

FIGURE 6.2: Relationship between mean egg length and mean offspring length. Each circle represents one clutch; Open circles and hatched linear trend line are for low, solid circles and solid linear trend line are for high. Low linear trend line: $y = 0.31x + 0.88$; $r^2 = 0.077$; High linear trend line: $y = 0.70x + 0.73$; $r^2 = 0.24$.

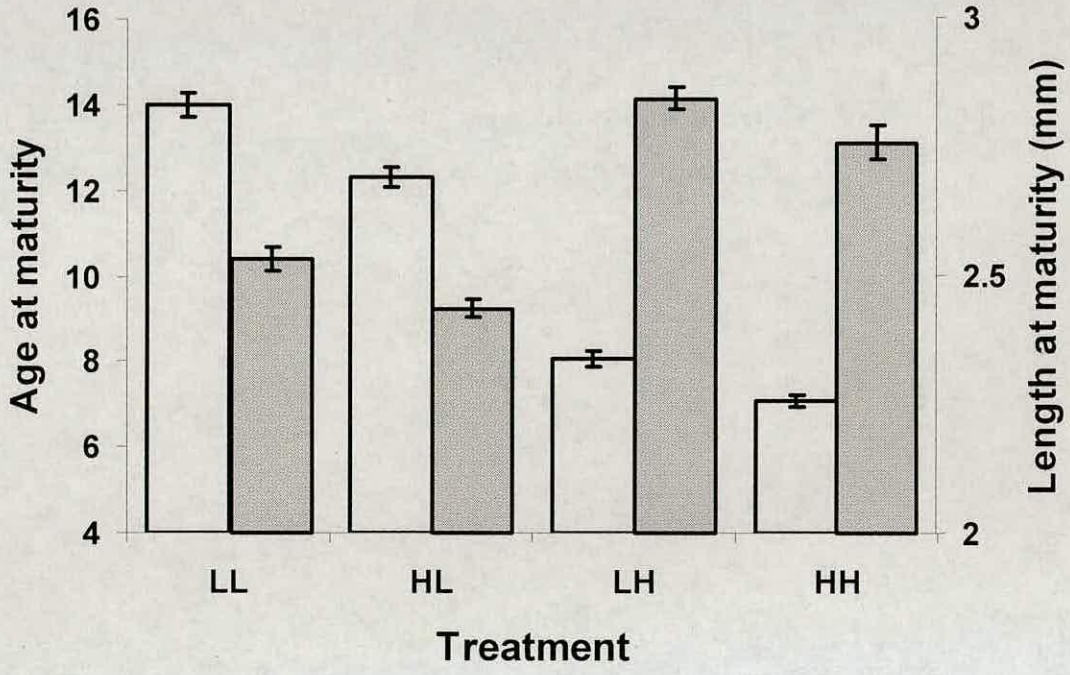

FIGURE 6.3: Relationship between treatment and age at maturity (light bars, measured as the day that eggs first appeared in the brood chamber), and treatment and size at maturity (dark bars, measured as body length when eggs first appeared in the brood chamber). *Daphnia* matured at earlier when current treatment = high, and when maternal treatment = high. *Daphnia* were larger at maturation when current treatment = high, and when maternal treatment = low. Note age at maturity is on the right-hand axis, while size at maturity is on the left. Age at maturity data are back-transformed means. Error bars are ±s.e.m.

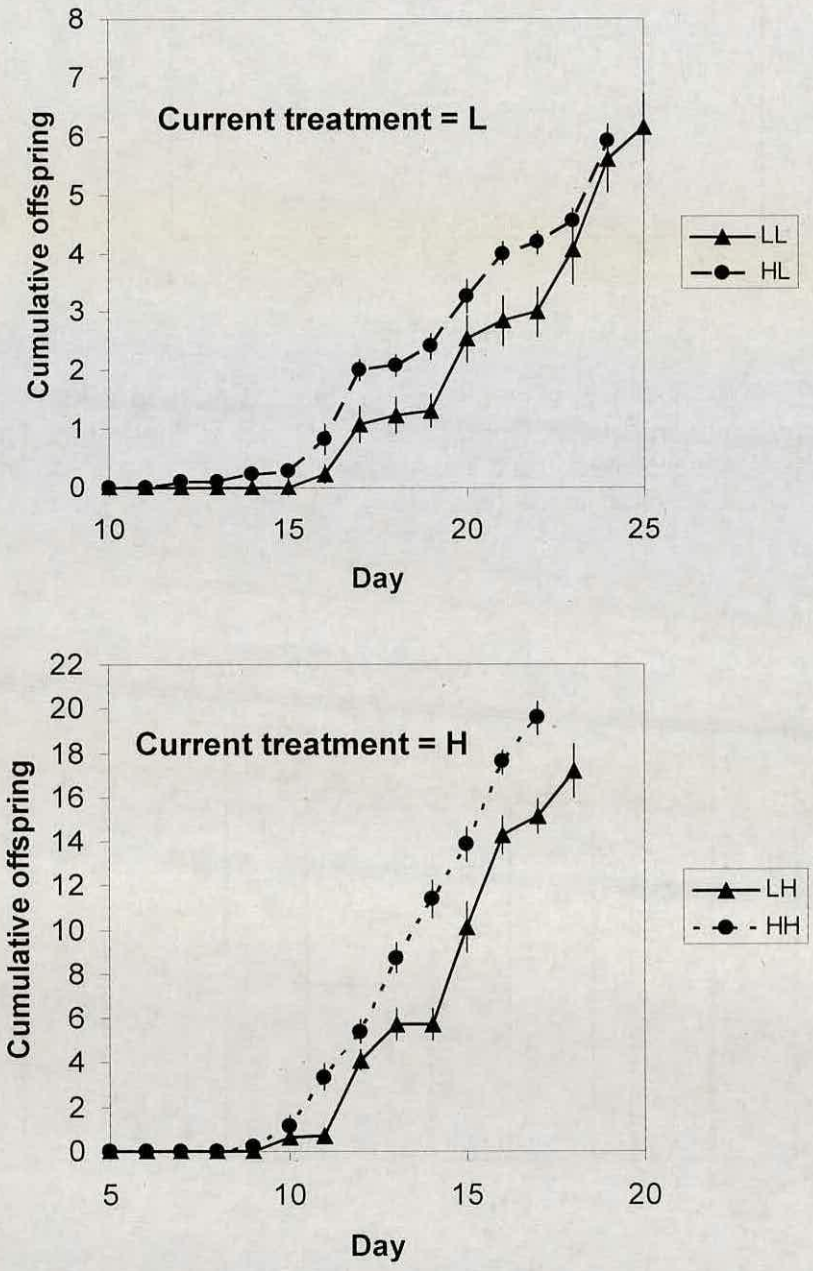

Figure 6.4: The cumulative number of offspring produced by each treatment. The top graph is for current treatment = L, the bottom is for current treatment = H. Within each current food treatment, *Daphnia* from low-food mothers had produced fewer offspring at any day post-hatching than *Daphnia* from high-food mothers. Error bars are ±s.e.m.

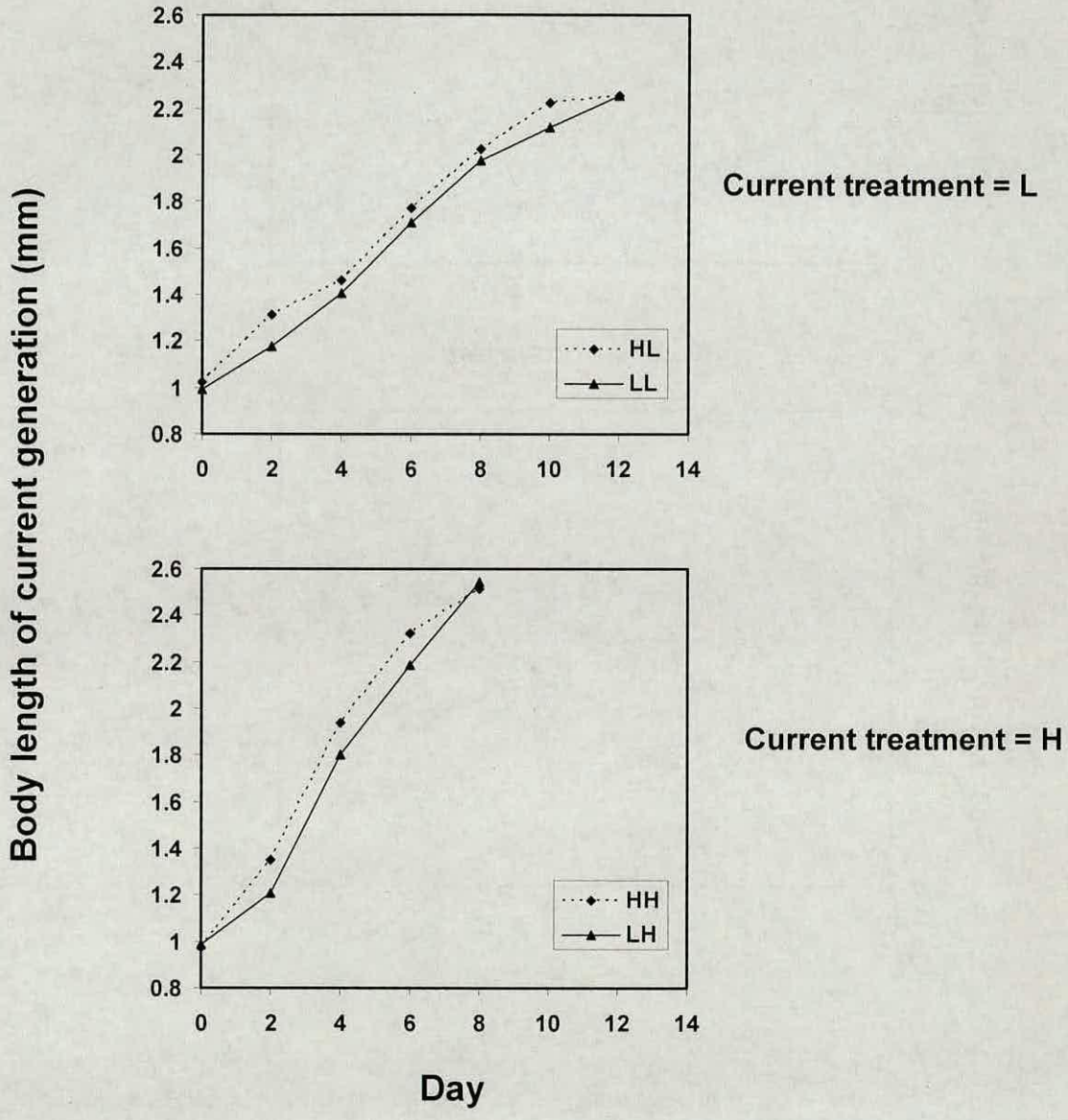

FIGURE 6.6: Mean body length, measured every 2 days after hatching, for each treatment. Within current treatment, those *Daphnia* whose mothers were at low food were smaller, but this was not statistically significant. Data are untransformed data.

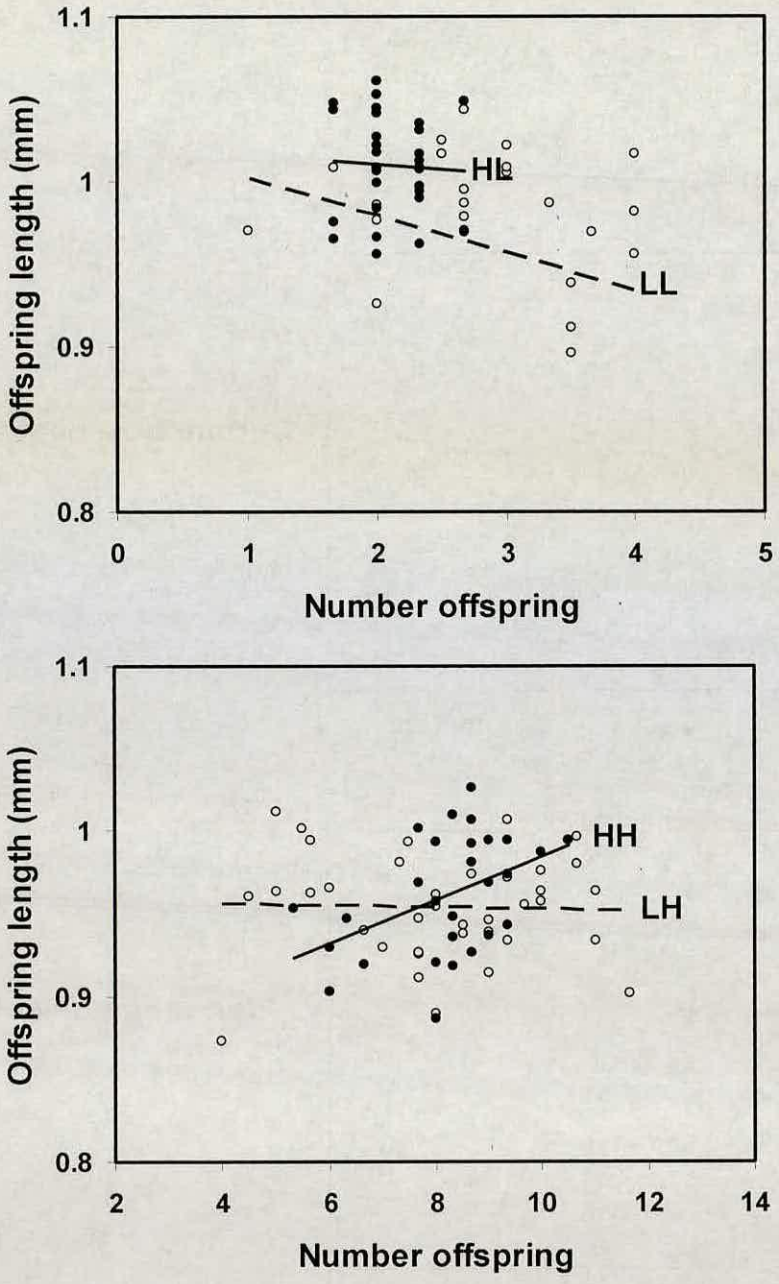

FIGURE 6.7: Number of offspring in a clutch versus mean offspring length for that clutch, by treatment. At current treatment = L, offspring from larger clutches were smaller. Clutch size and offspring size were positively related for the HH group, but there was no evidence of a relationship in the LH group. Each circle represents the offspring size and number of offspring for one *Daphnia* averaged over the first 3 clutches. The top graph is for current treatment = L, the bottom graph for current treatment = H. Open circles and broken linear trend line are for maternal treatment = L, Solid circles and solid linear trend line are for maternal treatment = H. Linear trend line equations: LL: $y = -0.023x + 1.03$, $r^2 = 0.03$; HL: $y = -0.006x + 1.02$, $r^2 = 0.003$; LH: $y = -0.0005x + 0.96$, $r^2 = 0.001$; HH: $y = 0.013x + 0.85$, $r^2 = 0.19$.

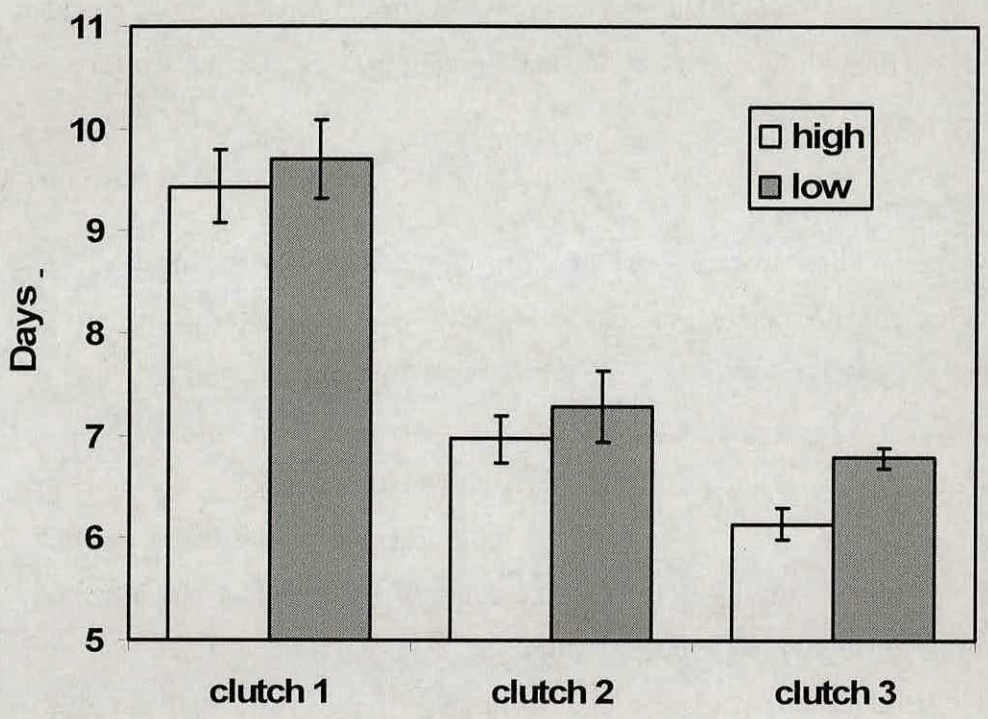

FIGURE 6.8: Mean number of days lived by offspring produced by mothers in high and low food environments, for the first 3 clutches.

6.5 Discussion

To examine how the environment of the mother affects the fitness of her offspring, we placed *Daphnia* offspring produced by mothers experiencing different food conditions (low or high) into one of 2 food treatments (same as maternal treatments) in a fully factorial design. As offspring size is generally negatively correlated with maternal food availability in *Daphnia* (table 5.1) and specifically in the clone of *D. magna* used here (figure 3.2), we expected that *Daphnia* produced by mothers in food-limited conditions would have higher fitness because they receive more resources from their mother (and are larger). We were therefore surprised to find

that (larger) offspring produced by mothers in low food took longer to reproduce, produced fewer offspring, and the offspring they did produce were smaller (figures 6.3-6.5). We also expected differences to be greater under food-limited conditions, as we expected that initial resources would be less critical when food was abundant. However, we found little evidence for a current x mother food interaction for any of our fitness measures.

Two previous studies have explored the effect of maternal food treatment on *Daphnia* reproduction, though these studies used *D. pulex* (Lynch & Ennis 1983; LaMontagne & McCauley 2001). We found that offspring from high food mothers grew faster (for a given current food treatment; figure 6.6), though this difference was not statistically significant. This agrees with the findings of Lynch and Ennis (1983), though LaMontagne & McCauley (2001) found the opposite (LL individuals grew faster than HL individuals). We found that offspring produced by low food mothers had lower early reproduction (for a given current treatment), as did Lynch and Ennis (1983; when the current food treatment =L). We also found that offspring produced by low food mothers were larger at maturity (for a given current food treatment), as did LaMontagne & McCauley (2001; when current food = L).

While we found no reproductive advantage to being born to a resource-limited mother (and therefore being larger), it might be that larger offspring have a survival advantage. Numerous studies have demonstrated that larger *Daphnia*, and/or *Daphnia* produced by food-limited mothers, have increased survival under food-limited or starvation conditions (Lynch & Ennis 1983; Tessier & Consolatti 1989; Gliwicz & Guisande 1992). Here, offspring from mothers at low and high food treatments were maintained at starvation conditions. For clutches 1-3, the offspring produced by the low-food mothers lived longer, though this difference was only statistically significant for clutch 3.

In all, our results, along with the results of others, suggest that *Daphnia* from food-limited mothers (hatching from larger eggs) have a survival advantage in starvation conditions, though the observed effect in this experiment were small. It might be that simple resource provisioning differences cannot explain the maternal effects observed in *Daphnia*, and offspring from mothers in low food environments are programmed to be cautious, and put more resources into survival rather than reproduction. This would explain both their increased survival and reduced fecundity. Future experiments should test for survival differences in low- and high-food environments, to ascertain whether there is indeed a trade-off between reproduction and survival.

Fitness is highly environmentally dependent—had we measured the fitness of our *Daphnia* in a very low food treatment, we might have found that the *Daphnia* produced by low-food mothers had higher fitness. Likewise, a fitness advantage of being produced by a food-limited mother might only be apparent later in life, after the period of time studied here. In addition, it has been suggested that fitness measures such as R_o (lifetime reproductive success), and r (rate of increase), estimated using life-history traits, do not necessarily predict the victor in competition assays in *Daphnia* (Mitchell 1997; Little *et al.* 2002). Unfortunately, competition trials between individuals of different sizes and with different maternal backgrounds would be challenging because it would be difficult to distinguish the individuals, and near impossible to determine the maternity of offspring (as all would be genetically identical).

Grand-maternal effects in *Daphnia*?

We found that the *Daphnia* provisioned resources to their offspring differently depending on their current food environment and the food environment of their mother, with HL individuals producing the largest offspring, LH producing the smallest, and LL and HH being in-between and similar to each other (figure 6.5). If this size difference translates into differences in the characteristics of that offspring,

as it most likely will, this suggests that not only maternal environment, but also grand-maternal environment, can influence an individual's characteristics. *Daphnia* live in a seasonal environment, and any deterioration in environment from one generation to the next might signal the *Daphnia* to produce more 'hardy' offspring to survive difficult periods (such as winter). Here, HL individuals produced larger (and presumably more starvation-resistant) offspring than LL individuals. Likewise, HL *Daphnia* are more likely to produce resting eggs than LL *Daphnia* (LaMontagne & McCauley 2001), and *Daphnia* whose mothers experience a shortened photoperiod (presumably signifying the coming of winter) are more likely to produce resting eggs which are resistant to environmental extremes (Alekseev & Lampert 2001).

Trade-off between clutch size and offspring size

Our study also allowed us to look for evidence of a trade-off between clutch size and offspring size. Given that a mother has a fixed amount of resources for reproduction, if she increases the amount of resources she provisions to each egg (i.e., makes each egg larger), she must then decrease the number of eggs she produces, and vice versa (Smith & Fretwell 1974; Roff 1992; Stearns 1992). It has, however, been notoriously difficult to observe such an effect in practice. Because of genetic or historical differences, certain individuals are generally fitter, and therefore perform better in all components of fitness (i.e., 'fitter' individuals produce both more and bigger offspring Reznick *et al.* 2000).

Clonal organisms such as *Daphnia* are well-suited to studying this problem because all effects of genetic background are eliminated. Glazier (1992) hypothesized that one would find a negative trade-off between offspring number and size in *Daphnia* that is more pronounced at low food levels (though he did not find support for this in *D. magna*). Here, we supported Glazier's (1992) hypothesis, in that offspring size and number were negatively correlated at low food, but a positive correlation, or no relationship, was found at high food (figure 6.7). Ebert (1993) found a similar result (using *D. magna*).

Conclusions

We found that offspring hatching from larger eggs produced by mothers in a food-limited environment reproduced later, and produced fewer, smaller offspring. Previous studies have found that larger neonates, or those neonates produced by food-limited mothers, have increased survival under low-food or starvation conditions (Lynch & Ennis 1983; Tessier & Consolatti 1989; Gliwicz & Guisande 1992). Overall, this suggests that offspring from food-limited mothers are programmed to be more cautious, investing resources into survival rather than reproduction. We also found evidence that offspring size is in part dependent on the conditions of the grandmother, suggesting that not only maternal effects, but grand-maternal effects might be important in *Daphnia*. Lastly, we found evidence of a trade-off between clutch size and clutch number, but only in food-limited conditions.

CHAPTER 7

HOST IMMUNE STATUS AFFECTS MATURATION TIME IN TWO NEMATODE SPECIES—BUT NOT AS PREDICTED BY A SIMPLE LIFE-HISTORY MODEL

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7.1 Abstract

In theory, the age at which maturation occurs in parasitic nematodes is inversely related to pre-maturational mortality rate, and cross species data on mammalian nematodes is consistent with this prediction. Immunity is a major source of parasite mortality and parasites stand to gain sizeable fitness benefits through short-term adjustments of maturation time in response to variation in immune-mediated mortality. The effects of thymus-dependent immune responses on maturation in the nematode parasites *Strongyloides ratti* and *Nippostrongylus brasiliensis* were investigated using congenitally thymus-deficient (nude) rats. As compared with worms in normal rats, reproductive maturity of parasites (presence of eggs *in utero*) in nude rats occurred later in *S. ratti* but earlier in *N. brasiliensis*. Immune-mediated differences in maturation time were not associated with differences in worm length. Thymus-dependent immunity had no effect on pre-maturational mortality. Results are discussed in relation to theoretical expectations and possible explanations for the observed patterns in parasite maturation.

7.2 Introduction

For most animals, life begins with a period of pre-maturational somatic growth, the duration of which can have a large impact on fitness. For example, generation time, mortality, body length and fecundity are themselves often functions of maturation time. The age of reproductive maturity is therefore of central biological importance and likely to be a major target of natural selection (Roff 1992; Stearns 1992).

For parasitic nematodes, an optimality model of maturation time has been developed based on the relationship of body size and fecundity (Gemmill *et al.* 1999). According to this model, extended growth before reproduction results in larger size which enhances fecundity, but also entails a heightened risk of death prior to reproduction. Thus, natural selection favours smaller, more rapidly maturing worms when the resulting increase in survival outweighs size-related reductions in fecundity. Conversely, when pre-maturational mortality is sufficiently low, the fecundity benefits of large size tip the balance in favour of delayed maturity. This model explains about half the variation in maturation time across a wide range of mammalian gastrointestinal nematodes. It might also explain variation in maturation time within species of nematodes (Gemmill *et al.* 1999).

In some free-living species, individuals adjust maturation time adaptively in response to environmental variation. For example, *Daphnia* (small freshwater crustacea) adjust their age and size at maturity in the presence of a predator, and are even able to discriminate between predators that prey on *Daphnia* of different sizes (and presumably of different ages). *Daphnia* exposed to chemicals from predators that prey on small *Daphnia* mature later and larger, while chemicals from predators that prey on large *Daphnia* result in earlier maturation at a smaller size (Weider & Pijanowska 1993; Stibor & Luning 1994). This is a phenotypic response and the nature and magnitude of the response can differ between *Daphnia* clones (Weider & Pijanowska 1993). Theoretical studies show that such a capacity can evolve where environmental variability has large effects on fitness and where individuals have

reliable cues that indicate the nature of that variability (e.g., Moran 1992; Scheiner 1993).

Within a host population, immuno-competence varies with factors such as nutritional status, age, pregnancy and stress (e.g., Anderson 1988; Stear & Wakelin 1998). Consequently, the life expectancy of a developing nematode is likely to depend very much on the type of host in which it finds itself. A worm that could assess the likely efficacy of the immune system of a host, and vary its life-history accordingly, could maximise its lifetime reproduction whatever the immune-competence of the host. In principle, components of the host immune system could provide the necessary cues. There is evidence that components of the host immune system might play a role in controlling development in schistosomes (Amiri *et al.* 1992; Davies *et al.* 2001; Ravindran 2001). The nematode *Caenorhabditis elegans* can enter a non-feeding, stress resistant stage, called a dauer larva, which can survive long past the typical *C. elegans* lifespan (Riddle & Albert 1997; Golden & Riddle 1982 and references therein). Many nematodes of veterinary importance (e.g. *Ostertagia spp., Haemonchus contortus, Trichostrongylus spp.*), can likewise enter a diapause state, in response to cool temperature or other environmental factors (Anderson 1992 and references therein).

The thymus-dependent immune system can substantially decrease *Strongyloides ratti* and *Nippostrongylus brasiliensis* reproduction and/or survival (Kassai 1982; Dawkins *et al.* 1982 and references therein; Smith *et al.* 1991; McKay *et al.* 1995; Gemmill *et al.* 1997). Consequently there should exist strong pressure to optimise life-history parameters in line with parasite mortality resulting from the host immune response. Can nematodes respond to the presence of the host immune system as *Daphnia* responds to the presence of predators?

Here we investigate whether and how nematode maturation time is dependent on host immune status in *Strongyloides ratti* and *Nippostrongylus brasiliensis*, two

intestinal nematode parasites of rats. We make use of congenitally athymic (nude) rats, which are incapable of mounting thymus-dependent immune responses. We quantify variation in parasite establishment, maturation time and body length to explore the following questions: Do nematode parasites delay maturation in immuno-deficient hosts? If so, is there a size benefit associated with the delay?

7.3 Materials and Methods

Study organisms

Strongyloides ratti and *Nippostrongylus brasiliensis* are gastrointestinal nematodes that are natural parasites of rats. Adult parasites of both species inhabit the mucosa of the small intestine. In the intestine, *S. ratti* are exclusively parthenogenetic females (Viney 1994), while *N. brasiliensis* reproduce sexually (Anderson 1992). Eggs of both species are shed into the intestine and pass with host faeces to the external environment where further development takes place resulting in infective third stage larvae (iL3s). When an iL3 comes into contact with a rat, it burrows through host skin and migrates to the intestine. The majority of worms have arrived at the gut by 50 hours (*N. brasiliensis*) or 70 hours (*S. ratti*) post skin penetration (Tindall & Wilson 1990).

Methods

Infections:

For both *Strongyloides ratti* and *Nippostrongylus brasiliensis* experiments, 100 iL3s were counted under a dissecting microscope and administered subcutaneously in saline (0.80% w/v NaCl). The syringe and needle were then flushed with saline, and the number of remaining worms counted. In this way, a precise inoculum size could be calculated.

Strongyloides ratti—The isofemale line ED5 (Viney 1996) was used and was maintained by serial passage in Wistar rats (B & K Universal, UK). Experimental animals were female rats, aged 6-10 weeks (Harlan, UK). Fifteen nude (Hsd:rnu/rnu) and fifteen heterozygous (Hsd:rnu/+) rats were infected with 100 iL3s as described above. We henceforth refer to the heterozygous rats as 'normal' rats, as they have fully functioning immune systems. To sample adult worms in the gut, five nude and five normal rats were euthanised on each of day 4, 5 and 6 post-infection (PI). The experiment was carried out in two experimental blocks, the first block consisting of 2 normal and 2 nude rats euthanised on each of days 4, 5, and 6 PI, and the second block consisting of 3 normal and 3 nude rats euthanised on each of days 4, 5, and 6 PI.

Nippostrongylus brasiliensis—Parasites were obtained from R.M. Maizels, University of Edinburgh, and maintained in female nude rats (HsdHan:rnu/rnu; Harlan, UK). Experimental animals were female rats, aged 9 weeks (Harlan, UK). Fifteen nude (HsdHan:rnu/rnu) and fifteen normal (HsdHan:rnu/+) rats were infected with 100 iL3s as described above. To sample adult worms in the gut, five nude and five normal rats were euthanised on each of day 5, 6 and 7 PI. This experiment was not done in blocks.

Faecal egg outputs:

Strongyloides ratti—To measure parasite reproductive output, faeces from the animals euthanised on day 6 PI were collected overnight on days 3 and 4 PI. Faecal cultures were prepared as described by Viney et al. (1992). After two days incubation at 25°C free-living stages were washed from culture plates, collected and counted under a binocular microscope as described by Gemmill et al. (1997).

Nippostrongylus brasiliensis—To measure parasite reproductive output, faeces were collected from every rat overnight on days 5, 6 and 7 PI. Egg output per night was estimated using a modified McMaster's technique.

Adult worms:

Strongyloides ratti—Intestinal parasite numbers (worm burdens) were determined as described in Gemmill, Viney & Read (2000). Briefly, the method is as follows. Immediately after rat death, the small intestine was excised, opened longitudinally and rinsed briefly in tap water. Each small intestine was then divided into three approximately equal parts and each part incubated separately in saline (0.8% w/v NaCl solution). After two hours incubation at 37°C, each portion of small intestine was vigorously rinsed and backwashed with fresh saline in order to detach any remaining parasites. Recovered parasites were killed and straightened by immersion in boiling formalin solution (10% formal saline). Parasites were then transferred to a droplet of glycerol on a microscope slide, encircled with fine ground glass, covered with a cover slip and sealed with a polyurethane-based sealant.

Nippostrongylus brasiliensis—Intestinal parasites were recovered as described in Kassai (1982). Briefly, the method is as follows. The small intestine of each rat was removed immediately after death and cut into three approximately equal sections. Each section was then cut into three pieces, slit along its length and placed in a gauze sack. The sack was suspended in saline in a test tube and incubated at 37°C for 2 hours. Worms migrated through the gauze and collected at the bottom of the tube. Recovered parasites were killed and fixed by immersion in boiling formalin solution (7% formalin). They were transferred to anhydrous glycerine according to Seinhorst's (1959) method as modified by De Grisse (1969). The worms were then placed in a drop of glycerine on a slide and sealed with wax.

Parasite measurements:

Parasites of both species were examined under a binocular microscope. Sex (for *N. brasiliensis*) and presence or absence of eggs *in utero* were recorded for each worm. The body length of each worm was measured using the PC_IMAGE software package (version 2.2.01:Foster Finlay Associates, UK) and a JVC video camera module (model TK 1270).

Statistical analyses

Worm measurements were averaged over rat to avoid pseudoreplication. The SAS System: Release 8.0 (SAS Institute) was used for all statistical analyses. Data were analysed using analyses of variance (ANOVAs), except for proportion data (worm sex ratio and the proportion of female worms with eggs *in utero*) which were analysed using the Wald chi-squared statistic (Q_W) from the proc logistic command in the SAS system. Host immune status, day PI, initial inoculum size, gut worm burden and experimental block were included in maximal models where appropriate. Significant effects involving block (replicate) are thus controlled for, but they are of little intrinsic interest in their own right, so that they are reported only where they qualitatively affect the conclusion. Where possible, all two- and three-way interactions were also included in maximal models. Non-significant terms ($P>0.05$) were eliminated from models using backwards elimination (Crawley 1993).

7.4 Results

Strongyloides ratti

Inoculum sizes ranged from 88 to 100 larvae with a mean of 94.8 and did not differ with host immune status, day PI or the interaction between the two ($P>0.05$). One of the nude rats scheduled to be euthanised on day 6 PI died of unknown causes prior to being euthanised; this rat was excluded from analyses. A total of 499 worms were included in this study. An attempt was made to measure every worm from every rat,

however, because some of the worms were broken or too twisted to measure accurately, approximately 45% could not be measured.

Reproduction:

Larvae were first detected in the faeces of all rats on day 4 PI. Worm output on day 4 PI was lower in nude rats as compared to normal rats, but this difference was non-significant ($F_{1,7}$=3.57, P=0.10). The proportion of egg-bearing worms in nude rats was significantly less than that in normal rats, indicating a delay in the onset of reproductive maturity in nude rats (Q_W=5.28, df=1, P=0.022; figure 7.1A). Nearly all worms (>99%) recovered on days 5 and 6 PI had eggs *in utero*.

Adult worm recovery:

Worm burdens increased with day PI faster in nude rats than in normal rats (day PI: $F_{2,17}$=6.04, P=0.010; host immune status: $F_{1,17}$=0.38, P=0.54; host immune status by day PI interaction: $F_{2,17}$=4.43, P=0.028; figure 7.1C).

Average worm length increased with day PI ($F_{2,24}$=31.92, P<0.0001; figure 7.1E). Parasite body length was not significantly affected by host immune status (host immune status: $F_{1,23}$=0.50, P=0.49; host immune status by day PI interaction: $F_{2,21}$=0.03, P=0.97; figure 7.1E).

Nippostrongylus brasiliensis

Inoculum sizes ranged from 80 to 107 larvae with a mean of 97.23 and did not differ with host immune status, day PI or the interaction between the two (P>0.05). A total of 1099 worms were included in this study. An attempt was made to measure every worm from every rat, however, because some of the worms were broken or too twisted to measure accurately, approximately 10% could not be measured.

Reproduction:

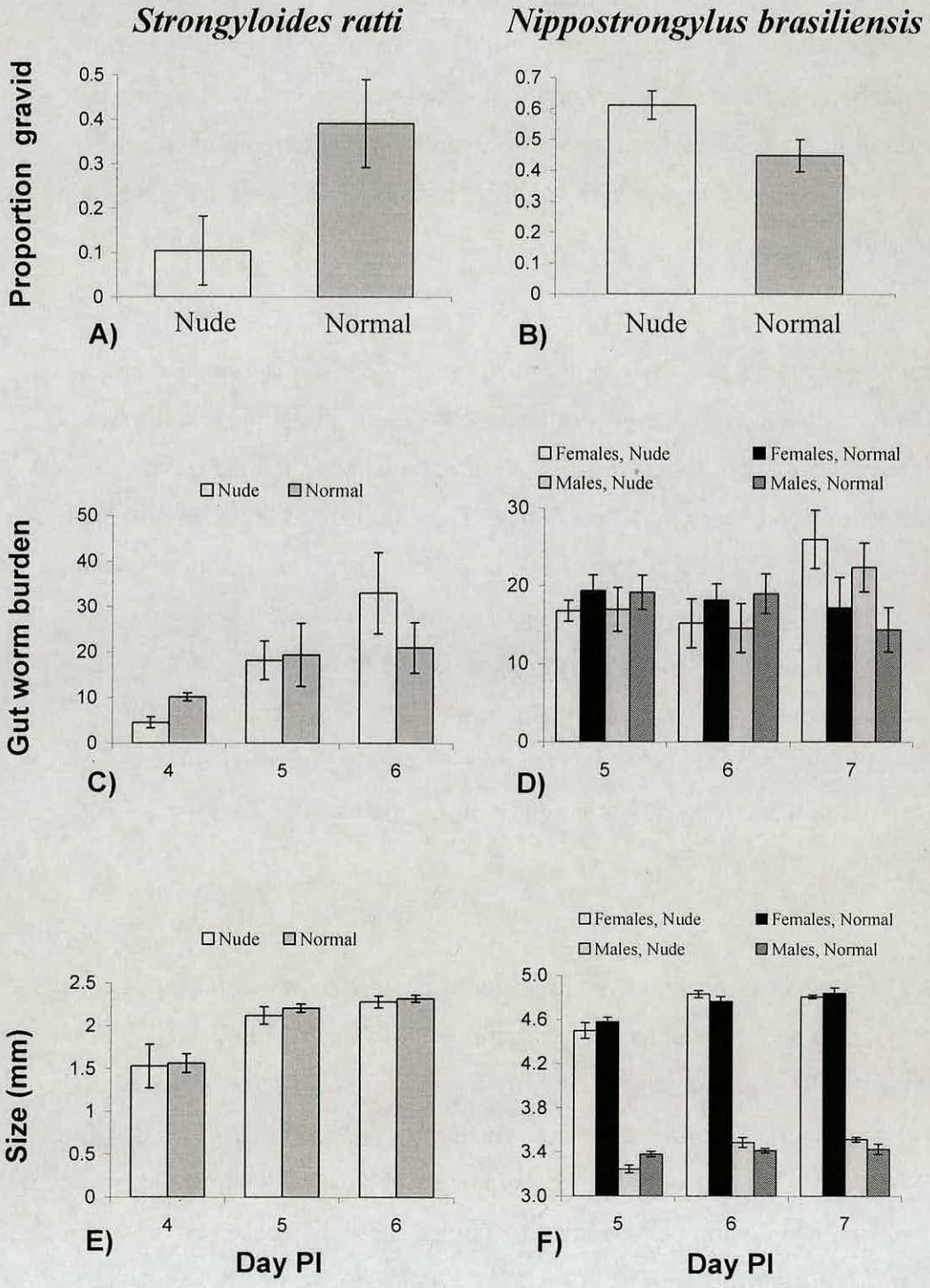
Eggs were first detected in the faeces of all rats on day 6 PI. Nude and normal rats had similar egg outputs on day 6 PI ($F_{1,18}$=0.0, P=0.97); but nude rats expelled more eggs on day 7 PI ($F_{1,7}$=13.8; P=0.0075). On day 5 PI, a higher proportion of female worms were gravid in nude rats than normal rats (host immune status: Q_W=5.51, df=1, P=0.019; figure 7.1B). By day 6 PI, nearly all female worms (96%) had eggs *in utero*.

Adult worm recovery:

Of all the *Nippostrongylus brasiliensis* recovered in this study, 85.6% were in the anterior, 13.3% were in the middle and 1.1% were in the posterior third of the small intestine. Fifty-two percent of recovered worms were female. Sex ratios did not vary significantly with day PI, host immune status or the interaction between the two (P>0.05). There was a slight decrease in gut worm burdens (total number of male and female worms) in normal rats as the infection progressed; this trend was not seen in nude rats (host immune status by day PI interaction: $F_{2,23}$=5.9, P=0.0084; figure 7.1D).

Female worm length increased with day PI, but was not affected by host immune status (day PI: $F_{2,26}$=26.5, P<0.0001; host immune status: $F_{1,25}$=0.05, P=0.83; host immune status by day PI interaction $F_{2,23}$=1.7, P=0.20; figure 7.1F). Male worm length increased with day PI, with those in nude rats increasing in length faster than those in normal rats (day PI: $F_{2,24}$=12.6, P=0.0002; host immune status: $F_{1,24}$=0.1, P=0.75; host immune status by day PI interaction $F_{2,24}$=6.6, P=0.0051; figure 7.1F).

Figure 7.1: White bars represent parasites from nude rats; black bars represent parasites from normal rats. Graphs A and B show the proportion of worms with eggs in utero day 4 (graph A, Strongyloides ratti) or day 5 (graph B, Nippostrongylus brasiliensis) for nude and normal rats (averaged over rat). Graphs C and D show the number of worms per rat on days 4, 5 and 6 PI (graph C, Strongyloides ratti) or 5, 6 and 7 PI (graph D, Nippostrongylus brasiliensis) for nude and normal rats. Graphs E and F show the mean body length (mm) of adult worms on day 4, 5 and 6 PI (graph E, Strongyloides ratti) or 5, 6 and 7 PI (graph F, Nippostrongylus brasiliensis). Each bar in graphs A-F is the mean of n=5 rats, except for Strongyloides ratti, nude, day 6 PI which is the mean of 4 rats. Errors are +1 s.e.m.


Strongyloides ratti
Nippostrongylus brasiliensis
Proportion gravid
0.5
0.4
0.3
0.2
0.1
0
A)
Nude
Normal
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
B)
Nude
Normal
Gut worm burden
Nude
Normal
50
40
30
20
10
0
C)
4
5
6
Females, Nude
Females, Normal
Males, Nude
Males, Normal
30
20
10
0
D)
5
6
7
Size (mm)
Nude
Normal
2.5
2
1.5
1
0.5
0
E)
4
5
6
Day PI
Females, Nude
Females, Normal
Males, Nude
Males, Normal
5.0
4.6
4.2
3.8
3.4
3.0
F)
5
6
7
Day PI

7.5 Discussion

Gemmill *et al.* (1999) predicted that individual parasitic nematodes stand to gain sizeable fitness benefits by adjusting maturation time in line with prevailing levels of immune-imposed, pre-maturational mortality. Where such mortality is high, more rapid maturation can enhance fitness by maximising survival to reproductive age. Where it is low, extended growth will pay off as increased size resulting in increased egg production.

In this study, exposure to the thymus-dependent immune system did indeed affect *Strongyloides ratti* and *Nippostrongylus brasiliensis* maturation time, but the two species experienced opposite responses. *S. ratti* matured later in nude rats than in normal rats (figure 7.1A), as found previously (Dawkins *et al.* 1982; Gemmill *et al.* 1997; Gemmill *et al.* 2000). Conversely, *N. brasiliensis* matured later in normal rats (figure 7.1B). The alterations in the timing of reproduction did not have any subsequent effect on worm length in either species (figure 7.1E and 7.1F). Thus, our study does not support our hypothesis that parasites experiencing less immune-dependent mortality mature later to increase size and optimise reproduction: *N. brasiliensis* actually matured earlier and the delay in maturation of *S. ratti* did not result in larger worms.

The model assumes that negligible worm growth occurs after maturation. We used the length of iL3s and the average female worm length on day 4 PI (*Strongyloides ratti*) and day 5 PI (*Nippostrongylus brasiliensis*) to estimate the average increase in length per worm per day before maturation (maturation was defined as occurring on day 5 PI for *S. ratti*, on day 6 PI for *N. brasiliensis*). We then calculated the average increase in length per worm per day post-maturation (growth between days 5 and 6 PI for *S. ratti*, between day 6 and 7 PI for *N. brasiliensis*). We found that the pre-maturational growth (mm/day) was at least three (*S. ratti*) to ten (*N. brasiliensis*) times greater than the post-maturational growth, supporting our assumption.

There are many possible reasons why this study did not support our life-history hypothesis, one of which is that the hypothesis is wrong. Alternatively, it might be that the worms are unable to appropriately sense the experimental immune environment we provided or appropriately adjust their life-history in it. Nude rats are presumably extremely rare in nature, indeed, if they occur at all, and it might be that the worms were confused by some novelty of their physiology. This could be tested by using other methods of immuno-suppression.

Another possibility is that the worms are actually extremely good at predicting their life expectancy and that our experimental manipulation does not in fact capture the situation in which alteration in maturation time would be favoured by natural selection. In these experiments, as in a previous experiment using *S. ratti* (Gemmill *et al.* 2000), there was no evidence that thymus-deficient hosts had lower pre-maturational mortality, although the increase in worms in nude rats with day PI might support this. In *S. ratti*, adult worm burdens on the first day of sampling were lower in nude rats, and in *N. brasiliensis* they were similar (figure 7.1C and 7.1D). Thus, the worms might have been correctly predicting no reduction in mortality in the nude rats. But if so, it begs the question of why maturation times were nonetheless altered. Moreover, the larval forms of these parasites are highly susceptible to immune response in hosts with prior exposure (Kassai *et al.* 1974 as cited in Kassai 1982; Gemmill *et al.* 1997). We believe that the observed lack of immune-mediated, pre-maturational parasite mortality is most likely to result from a lag between parasite invasion and host immune response in naïve hosts, rather than a lack of immune response to larval stages. Under more natural circumstances, a lack of thymus-dependent immunity should predict enhanced larval survival, and worms should delay reproduction accordingly.

Alternatively, the model might be too simple. For example, if post-maturational or size-dependent mortality are themselves important functions of maturation time, this might affect the nature of the optimal phenotypic response to shifts in the prevailing mortality schedule (Skorping & Read 1998). If so, quantitative success of the

Gemmill *et al.* model (1999) in predicting cross-species variation in age to maturity would presumably be enhanced by taking these complexities into account.

It might be significant that the highest worm counts for both parasite species occurred in nude rats on the last day of the experiment (figure 7.1C and 7.1D), and day PI was correlated with worm number for *N. brasiliensis* in nude rats (*N. brasiliensis*: $F_{2,11}=6.1$, $P=0.03$; *S. ratti*: $F_{1,12}=2.3$, $P=0.15$). It is possible that the worms in nude rats experienced slowed migration, development and reproduction in the absence of immune cues; this would not be the first such finding in a helminth parasite (Amiri *et al.* 1992; Ravindran 2001; Davies *et al.* 2001). If this is the case, no appreciable size benefit was associated with the delay, and therefore no evidence of an adaptive advantage, was detected (figure 7.1E and 7.1F). The alterations in time of maturity in *S. ratti* and *N. brasiliensis* in nude rats could be a pathological consequence of life in an novel environment for which they were unprepared by evolution; alternatively, the alterations might represent adaptive short-term adjustments of the developmental schedule, the fitness consequences of which remain to be determined.

CHAPTER 8

A PARASITIC NEMATODE IS NEGATIVELY IMPACTED BY NEMATODE DENSITY—BUT NOT IN IMMUNO-DEFICIENT HOSTS

8.1 Abstract

Nematode size and fecundity are important to disease pathology and epidemiology. At higher nematode densities, nematode size and (per worm) fecundity are generally lower. This might be because the host immune response is proportional to worm number, and the host immune response regulates nematode size and fecundity. Conversely, it might be the result of direct competition between the worms. To distinguish between these two hypotheses, the effect of worm density on worm length and fecundity in *Nippostrongylus brasiliensis* in normal rats and thymus deficient (nude) rats were compared. Female worm size decreased with increasing worm density in normal rats, but was constant over worm density in nude rats. Male worm size was not significantly affected by number of worms in nude or normal rats. Fecundity was decreased at higher worm densities in normal rats, but increased at greater worm densities in nude rats. This is evidence that the immune response increases when more worms are present, and that it limits nematode size and fecundity.

8.2 Introduction

Nematode parasites are a major cause of disease in humans, and result in significant economic losses to domestic livestock through lowered productivity and the costs of drug therapy. There is evidence that nematode size can influence disease pathology—the bigger the nematode, the worse the disease symptoms (e.g. Stear *et al.* 1999). Parasitic nematodes (of the same species) show much variation in adult body size; the cause of this variation has yet to be adequately explained (Kloosterman *et al.* 1989; Kloosterman *et al.* 1990; Stear *et al.* 1999; Davies *et al.* 2001). While worm genetics undoubtedly plays a part, in the sheep parasite *Ostertagia circumcincta* host genetics is a better predictor of worm size than worm genetics (Stear *et al.* 1997; Stear *et al.* 1999). Therefore a nematode's environment must play a large role in determining worm size.

In nematodes, worm size is negatively correlated with worm density (e.g. Scott & Lewis 1987; Stear *et al.* 1995; Tompkins & Hudson 1999). Likewise, nematode fecundity is negatively correlated with parasite density (e.g. Keymer 1982; Scott & Lewis 1987; Sinniah & Subramaniam 1991; Paterson & Viney 2002; Ryder & Griffin 2003), probably because there is a strong relationship between worm size and per capita fecundity (measured as egg output or eggs *in utero*; Sinniah & Subramaniam 1991; Stear *et al.* 1995; Stear *et al.* 1997; Tompkins & Hudson 1999; Stear & Bishop 1999).

Previous studies suggest that observed density effects are due to an increased host immune response in the presence of more worms (Stear *et al.* 1995; Stear *et al.* 1997; Stear *et al.* 1999; Paterson & Viney 2002); but it is also possible that interactions between worms (e.g. competition for resources or mates) could affect worm size and/or fecundity. It is important that we distinguish between these two possibilities for two reasons. First, immune-mediated density-dependent effects will depend on previous exposure, while density-dependent effects resulting from competition

between worms will depend solely on current infection burden. Current models of nematode epidemiology incorporate acquired immunity (Roberts & Grenfell 1991; Woolhouse 1992; Smith & Grenfell 1994; Grenfell *et al.* 1995; Woolhouse 1998); but there is a dearth of accurate information to estimate the parameters modelling immunity. Second, as there is evidence that worm size and pathology are related for at least one nematode species (Stear *et al.* 1999), an understanding of the relationship between the immune system and worm size is necessary to better predict the efficacy of vaccinations and anthelmintic treatments within an individual host.

In this study, we explore the relationship between nematode density, nematode body size and per capita fecundity in a parasitic nematode. We make use of nude rats, which are congenitally athymic and therefore unable to mount a thymus-based immune response. By comparing nematodes in nude and normal rats, we can better understand whether observed density dependence results from the host immune response or interactions between worms.

8.3 Materials and Methods

Study organism and methods

This study reports further on the results of the experiment described in Chapter 7 for *Nippostrongylus brasiliensis*, therefore see Chapter 7 for Materials and Methods.

Statistical analyses

The SAS System: Release 8.0 (SAS Institute) was used for all statistical analyses. Per capita egg production was determined by dividing each rat's overnight faecal egg output by the number of gravid females recovered from that rat's intestine the next day. Analyses of covariance (ANCOVAs) were used to determine whether host immune status and gut worm burden affected per capita egg production and worm size. Because rats were sacrificed over 3 days, date of sacrifice was included in the

maximal model for worm size, and the analysis for per capita fecundity was carried out separately for days 6 and 7 post infection (PI). Maximal models were: worm size = host immune status + gut worm burden + date + host immune status * gut worm burden; and per capita fecundity = host immune status + gut worm burden + host immune status * gut worm burden. Non-significant terms were dropped from models using backwards selection (Crawley 1993).

8.4 Results

As location in the gut can affect *Nippostrongylus brasiliensis* size (personal observation), and because this parasite prefers the upper third of the small intestine (Kassai 1982), only worms recovered from the upper third of the small intestine were included in analyses (86% of recovered worms were in the top third of the intestine).

Female worm length was negatively correlated with number of worms in the gut ($F_{1,27}$=8.1, P=0.008). Statistically, this effect did not differ by host immune status (host immune status: $F_{1,26}$=0.45, P=0.51; gut worm burden by host immune status interaction: $F_{1,25}$=0.28, P=0.6). However, when I analysed the host immune status' separately, females in normal rats decreased in length as gut worm burden increased ($F_{1,12}$=13.6, P=0.003), while female worm size in nude rats did not show any significant effect of worm density ($F_{1,12}$=4.5, P=0.06; figure 8.1). Male worm length was not affected by gut worm burden (gut worm burden: $F_{2,27}$=1.9, P=0.18; gut worm burden by host immune status interaction: $F_{1,25}$=1.6, P=0.22; figure 8.1). Eggs were first recovered from the faeces of all rats on day 6 PI. Per capita egg production did not significantly differ with immune status on day 6 PI (P>0.05). On day 7 PI, the host immune status by gut worm burden interaction was statistically significant (gut worm burden by host immune status interaction: $F_{1,6}$=14.8,

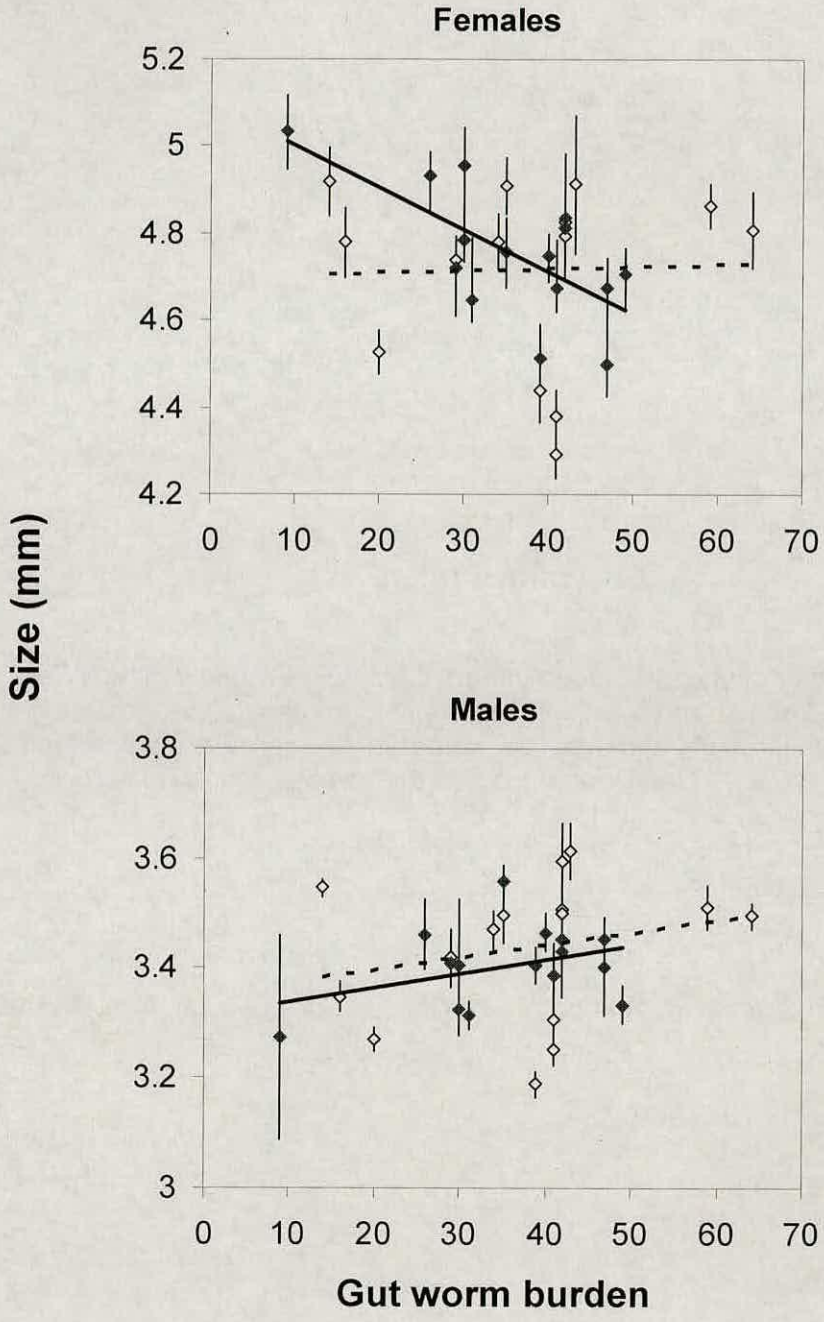


Figure 8.1: Female and male worm length versus gut worm burden. Each point is the mean worm length of worms from one rat. Closed diamonds and the solid regression line represent worms from normal rats (n=30; female: -0.0097x + 5.1, r^2 = 0.45; male: 0.0026x + 3.3, r^2 = 0.14). Open diamonds and the hatched regression line represent worms from nude rats (n=30; female: 0.0005x + 4.7, r^2 = 0.001; male: 0.0023x + 3.4, r^2 = 0.057). Errors are ±1 s.e.m. for each rat.

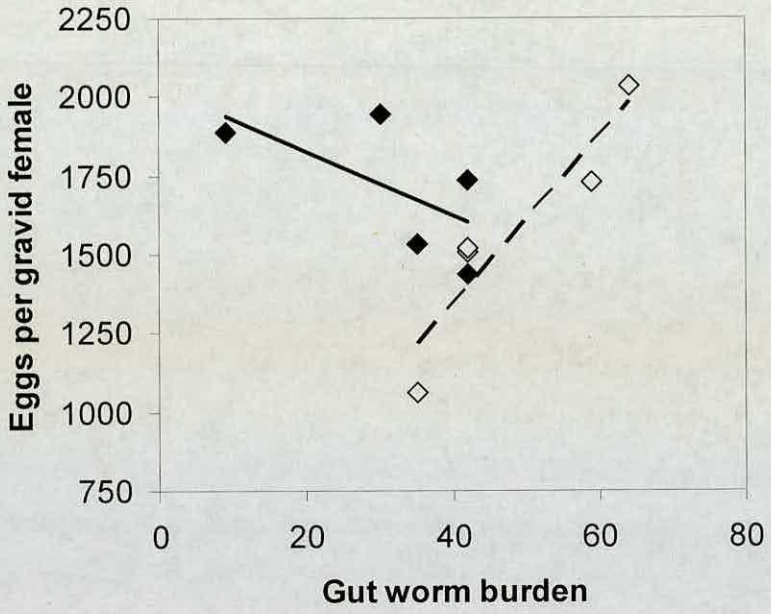


Figure 8.2: Number of eggs produced per gravid female overnight on day 7 PI versus gut worm burden. Each point is the mean number of eggs per gravid worm for one rat. Closed diamonds and the solid regression line represent normal rats ($y = -10.2x + 2031$; $r^2 = 0.39$). Open diamonds and the hatched regression line represent nude rats ($y = 26.6x + 281$, $r^2 = 0.87$).

P=0.009), as the number of eggs per gravid female increased with gut worm burden in nude rats ($F_{1,3}$=19.7, P=0.02), but decreased (though not significant statistically) with gut worm burden in regular rats ($F_{1,3}$=2.0, P=0.26; figure 8.2).

8.5 Discussion

This is the first study to directly explore both the impact of the thymus-based immune system and worm density on nematode size. Our results support previous reports of a negative correlation between worm length and worm density in immuno-competent hosts (e.g. Scott & Lewis 1987; Stear *et al.* 1995; Tompkins & Hudson 1999), but only for female worms. A negative body size effect of increased worm

density (in female worms) was observed in normal rats, but not in rats lacking a thymus-based immune system (figure 8.1; females). This suggests that the density-dependent size effect resulted from an increased immune response in the presence of more worms, rather than direct interactions between the worms themselves. Male worms are less immunogenic, and female worms are expelled faster than males (Kassai 1982); this might possibly explain why male worm size was not affected by worm density (figure 8.1; males). Wilkes *et al.* (2004) found that *Strongyloides ratti* recovered from nude rats early in infection (day 6 PI) and late in infection (day 43 PI) did not differ in size, but that worms in normal rats were smaller later in infection (as compared to earlier in infection, when they did not differ in size from those in nude rats). Wilkes *et al.*'s (2004) results further suggest that the host immune system limits worm size.

While not statistically significant, worm fecundity in normal rats was lower at higher worm densities, supporting evidence from previous studies that the host immune system regulates egg production, and that the immune response is increased when there are more worms (Stear *et al.* 1995; Stear *et al.* 1997; Stear *et al.* 1999). Increased worm density in the absence of a thymus-based immune response did not negatively impact fecundity; on the contrary, egg output increased significantly with total number of worms in nude rats (figure 8.2). This might be because increased worm density increases the likelihood of encountering a mate.

We know of only one previous study exploring nematode fecundity in relation to parasite density in nude and normal animals; this was a study on fecundity and survival on *Strongyloides ratti* in rats (Paterson & Viney 2002). As here, Paterson and Viney found that worm density and per-worm fecundity were negatively correlated in normal rats; however, nude rats had similar fecundities regardless of worm burden. They also found a negative relationship between worm density and survival in normal, but not nude, rats. Paterson and Viney's experiment covered a much greater range of nematode densities (10-1000 worms) than the study reported

here, and they found no negative impact of density on survival or fecundity in nude rats at any worm density studied.

One possible weakness of this study is that all rats were inoculated with (roughly) the same number of worms, so differences in worm burdens came about through differential establishment. Therefore, we cannot be sure that the patterns observed are not the result of confounding variables. For instance, if smaller worms were more likely to be killed by the immune system, we would expect larger worms in rats where more worms were killed by the immune system (i.e., 'normal' rats that had low worm burdens), as was observed. Therefore, future studies should examine worm size/fecundity in normal and immune hosts inoculated with differing numbers of nematodes. Future studies should also explore a larger range of worm densities, to determine if competition limits worm size and worm fecundity at higher worm densities.

In conclusion, this study was the first to explore how worm density affects worm size in nude and normal animals, and we found evidence that the host immune system limits both worm size and worm fecundity. We did not find evidence that direct competition between worms affects worm size and/or fecundity; however, worm densities in this study were low, and competition between worms might become apparent at higher densities. This study offers direct evidence that the immune system can limit nematode size and fecundity.

CHAPTER 9

GENERAL DISCUSSION

9.1 Summary

In this era of genomes, molecular markers, and microsatellites, the take-home message of my thesis is perhaps to remind us that organisms are not just a product of their genes, but are also shaped by their environment. Small differences in an individual's surroundings, or even its mother's surroundings, can profoundly affect when, how, and how successfully an animal reproduces. Using *Daphnia magna*, parasitoids wasps, and parasitic nematodes, I have explored these responses and attempted to explain them using adaptive reasoning, and/or mathematical modelling. When and how an animal reproduces is certain to have consequences for its fitness, and even the evolution of species. Specific conclusions from my work are discussed in the relevant chapters. Here, I briefly outline my findings, then discuss areas for future research.

9.2 Offspring resources: variability and fitness consequences

Main findings

While the trade-off between offspring size and number is one of the most studied areas of evolutionary biology, we still do not have a complete understanding of why offspring (egg) size variation exists within species. With this in mind, I tested a model predicting how range in offspring (egg) size is related to clutch size using two study organisms. In Chapter 3, I tested this model using a large number of genetically identical *Daphnia* in a laboratory setting, resulting in high power and low

variability from environmental effects. In Chapter 4, I used multiple species of parasitic wasp, allowing me to test the generality of the model. For both studies, as predicted, range in offspring (egg) size decreased with increasing clutch size. In *Daphnia*, I also found that mean egg size decreases with increasing clutch size.

In Chapter 5, I explored a modified version of the small clutch model first described by Charnov *et al.* (1995; Charnov & Downhower 1995) to examine the egg size variability and mean egg size patterns discovered in *Daphnia* (Chapter 3). I concluded that the observed negative relationship between egg size and clutch size could result from either a minimum viable egg size or from selection for larger eggs at lower resource levels. Additionally, I explored how this affects the invariant predicting the relationship between range in egg size and clutch size.

I then went on to determine how the differences in egg size in *Daphnia* (described in Chapter 3), translate into offspring fitness (Chapter 6). I hypothesised that larger offspring would be more fit, but (larger) offspring produced by low-food mothers took longer to reproduce, did not produce more offspring per clutch, did not produce larger offspring, and produced fewer offspring per unit time. This suggests that simple resource provisioning differences cannot explain the maternal effects observed in *Daphnia*. Past studies have found that larger *Daphnia* and those from low-food mothers are better able to survive low-food and starvation conditions (Lynch & Ennis 1983; Tessier & Consolatti 1989; Gliwicz & Guisande 1992), and we found some evidence of a slight increase in survival in those offspring born to mothers in low-food conditions. It might be that offspring produced by food-limited mothers are programmed to be cautious, and put more resources into survival rather than reproduction. However, fitness is difficult to measure, and more research needs to be done before conclusions can be drawn.

Future directions

There is an abundance of clutch size / offspring size data in existence for multiple and diverse species that would be ideal for testing the small clutch invariants examined here. Specifically, the plethora of clutch size / egg size data in birds, and seed number / seed size data in plants, are ripe for such analysis. Such studies would allow for an understanding of the generality of these models over multiple and varied taxa.

Egg size / offspring fitness curves have been worked out for very few species, and generally only in one environment. *Daphnia* are one of the few organisms in which a thorough fitness curve in multiple environments is feasible, and such an analysis would be highly useful for theoretical work (such as the analyses in Chapter 5).

The work presented in this thesis suggests that *Daphnia* in food-limited environments produce fewer offspring that are, on average, larger than offspring produced when food is abundant. However, those offspring are also highly variable in size. Some will be quite large and will likely out-compete the smaller *Daphnia* for the limited resources; I can envision direct competition experiments between *Daphnia* of different sizes/histories in the lab. This would generate parameter estimates with which one could easily model how these size differences would affect *Daphnia* population dynamics.

Lastly, while I attempted to test for the impact of maternal environment on offspring fitness, fitness is notoriously difficult to measure, as it is dependent on a multitude of variables. While I tested the survival under starvation conditions of offspring born to low- and high-food mothers, future tests should examine survival under multiple current food environments, to test for a trade-off between survival and reproduction. Additionally, fitness is highly environmentally-dependent, and future tests should examine maternal effects using a variety of different environmental variables, including food treatments, temperature, pheremones, and photoperiod. Such data

will help us to determine if, and under what environmental conditions, larger eggs are more fit. In addition, it will allow for an improved understanding of the possible mechanisms of maternal effects.

9.3 The immune system and parasite life-history traits

Main findings

In Chapters 7 and 8, I found that the life-history traits of intestinal nematodes were dependent on their environment. I found that parasitic nematodes matured at different rates in different host immune environments (Chapter 7) – one species as predicted by theory (*S. ratti*) and one in the opposite direction predicted by theory (*N. brasiliensis*). There were no associated size-differences between nematodes experiencing different immune regimes. It is possible that the worms are unable to sense the experimental immune environment I provided or appropriately adjust their life-history in line with it. Alternatively, it might be that the life-history model is too simple to predict life-history within a species, and a more complicated model taking into account the varied and complex life-histories of these parasites might be necessary. This study explored early, post-maturational growth in these species to a degree that had not been done previously.

Early in infection, nematode reproduction was negatively affected at high worm densities, but only in rats with a functioning immune system (Chapter 8). Specifically, higher nematode density was associated with lower per-capita egg production in rats with an intact immune system, but in those nematodes in rats lacking a thymus-based immune response, the opposite was the case. It is likely that the host immune system limits worm fecundity, and the host immune response increases with nematode density, explaining the negative correlation between worm density and fecundity in intact animals. More difficult to explain is the positive relationship between nematode density and fecundity in nude rats; it might be that the likelihood of encountering a mate increases with increasing nematode density.

Future directions

Theory predicts that nematodes should adjust age at maturity in line with host immune status – worms in hosts with weak immune systems should mature later. I tested for a phenotypic adjustment (Chapter 7) and found some support for this theory. Parasite mortality rates differ between hosts for a variety of reasons – vaccination, immuno-deficiency, drug therapy, genetics – and theory also predicts, if the environmentally-imposed nematode mortality rate is similar over many generations, the worms should evolve maturation time accordingly (Skorping & Read 1998; Gemmill *et al.* 1999; Read *et al.* 2000). The next step would be to test whether this theory holds on the evolutionary level: one could passage the worms through animals with different levels of immune response (perhaps nude, 'regular', and immunized rats) for many generations and observe whether and how their life-history traits evolve over time.

I found evidence of density-dependent effects in *Nippostrongylus brasiliensis* parasitizing 'regular', but not nude, rats (Chapter 8). This suggests that worm size and fecundity are limited by the host immune response. However, in our study, any differences in worm density were down to differences in establishment rate, so I could not rule out confounding variables. The next sensible experiment would be to infect nude and 'regular' rats with different numbers of worms, and compare worm size and fecundity. I attempted just such an experiment during my PhD. Unfortunately, my infections were (uncharacteristically) rejected early in infection, and I ran out of time before I could discover the cause of this.

A logical area of future nematode research would be to examine how the life-history and fitness differences described in this thesis will affect population dynamics. Specifically, current models of nematode epidemiology incorporate acquired immunity (Roberts & Grenfell 1991; Woolhouse 1992; Smith & Grenfell 1994; Grenfell *et al.* 1995; Woolhouse 1998); but there is a dearth of accurate information

to estimate the parameters modelling immunity. It would be useful to estimate model parameters for these models using the data from Chapters 7 and 8, along with those from other studies (e.g. Paterson & Viney 2002), to explore how the immune system might affect nematode population dynamics without necessarily killing the parasites.

9.4 Final thoughts

The work presented here, along with the work of others who study how organisms are affected by their environment, carry an important message. Everyone knows that humans are drastically altering the environment to the detriment of wildlife – bald eagles are unable to produce viable eggs because of DDT contamination, the oceans are covered in oil slicks and thousands of acres of rainforest have been reduced to vast wastelands. But these are only the obvious examples – what about the subtler impact that we might be having on the organisms around us? A slight change in the food availability of small freshwater crustaceans, resulting from nitrogen run-off from a farmer's field, for example, could impact reproductive strategy. This could, in turn, alter population dynamics, the effects of which might be seen throughout the food chain. Changes in the immune environment of a parasite, through vaccination or drug therapy, might cause that parasite to mature at a different rate, impacting pathology and transmission. While this thesis has not directly examined the implications that human actions have for the environment, I have shown that small changes in an organism's environment can impact on how, when, and how successfully it reproduces, often in ways that not obvious from the outset. These differences are likely to impact the population dynamics of that species, which could in turn affect food webs, ecosystems, and even the evolution of species. We would do well to keep this in mind.

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APPENDIX — Published papers arising from this thesis

M.A. Guinnee, S.A. West and T.J. Little. 2004. Testing small clutch size models with *Daphnia*. *The American Naturalist*, 163:880-887 (from Chapter 3).

M.A. Guinnee, A.W. Gemmill, M.E. Viney, B.H.K. Chan and A.F. Read. 2003. Host immune status affects maturation time in two nematode species—but not as predicted by a simple life-history model. *Parasitology*, 127:507-512 (from Chapter 7).

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Testing Small Clutch Size Models with *Daphnia*

Meghan A. Guinnee,* Stuart A. West,† and Tom J. Little‡

Institute of Cell, Animal, and Population Biology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, United Kingdom

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ABSTRACT: Life-history theory predicts that for small clutches, variance in egg size (between individuals) should decrease in a predictable invariant manner as clutch size increases. To test this, we studied *Daphnia magna* at 350 different food treatments and recorded the number of eggs and the volume of each egg for their first clutch. As predicted, we found that the relationship between clutch size and resources devoted to reproduction was linear, variance in egg volume decreased with increasing clutch size, and resources were shared relatively equally between the eggs in a clutch. However, we found that the rate at which the range of egg volumes decreased with clutch size was slower than predicted. We discuss possible explanations for this discrepancy, including a lower limit on the volume of eggs that are produced and selection for smaller eggs when food is abundant. Consistent with this, we found that mean egg volume decreased with increasing clutch size.

Keywords: clutch size, *Daphnia magna*, egg size, litter size, resource allocation, trade-off.

Examining how mothers allocate resources to their offspring can shed light on how natural selection shapes life histories (Roff 1992; Stearns 1992; Godfray 1994). Evolutionary models exploring optimal clutch (litter) size have typically used the approach described by Smith and Fretwell (1974), which models the trade-off between offspring size and offspring number assuming that mothers have a finite amount of resources available for each clutch and that better-provisioned offspring are fitter. However, because the predicted optimal clutch size is often not an integer value, these models must round to the nearest clutch size. Although this approach generally makes useful approximations at larger clutch sizes, it breaks down for smaller clutch sizes (Smith and Fretwell 1974). For example, if a mother has resources intermediate to those required for producing a clutch size of one or two, she then has to decide between producing one offspring that is larger than optimum size or two offspring that are smaller than optimum size. Recently, a number of theoretical models have addressed this problem and explicitly examined the consequences of small integer numbers for clutch size evolution (Ebert 1994; Charnov and Downhower 1995; Charnov et al. 1995; Downhower and Charnov 1998; West et al. 2001).

A novel and useful feature of these small integer models is that they make quantitative predictions that do not depend on difficult-to-measure life-history details and underlying trade-offs. Specifically, Charnov, Downhower, and colleagues have developed two life-history invariant rules. First, variance in offspring size (between clutches) is predicted to decrease with increasing clutch size, whereby the ratio of the range of offspring sizes for clutches of size i and j offspring is the reciprocal of the ratio of clutch sizes (Charnov and Downhower 1995; Charnov et al. 1995; West et al. 2001). This is given formally by

$$\frac{I_{\max i} - I_{\min i}}{I_{\max j} - I_{\min j}} = \frac{C_j}{C_i}, \tag{1}$$

where I is offspring size, C is clutch size, $I_{\max i}$ and $I_{\min i}$ are the maximum and minimum size of offspring in clutches of C_i young, i and j are clutches of different sizes, and $i = j + 1$. Figure 1 graphically depicts this relationship, which we refer to as Charnov et al.'s invariant rule. A decrease in between-clutch egg size variance was also predicted by Ebert (1994), though Charnov et al. (1995; Charnov and Downhower 1995) were the first to make the invariant predictions. The second invariant rule is that the increase in resources devoted to reproduction required to go from producing a clutch size of C to a clutch size of $C + 1$ is invariant, regardless of clutch size (Downhower and Charnov 1998). We refer to this as the Downhower and Charnov invariant rule.

The usefulness of these two invariant rules is that they are independent of underlying trade-offs, such as the re-

* Corresponding author; e-mail: meghan.gannon@ed.ac.uk.

† E-mail: stu.west@ed.ac.uk.

‡ E-mail: tom.little@ed.ac.uk.

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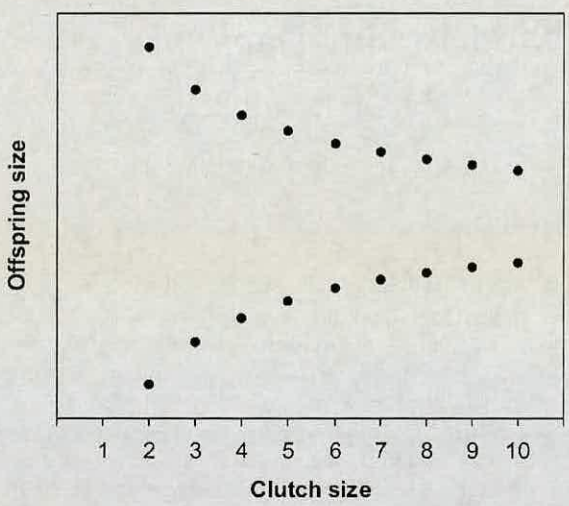

Figure 1: Predicted relationship between range in egg volume and clutch size (as described by eq. [1]). The upper point refers to the largest egg volume and the lower point to the smallest egg volume predicted for that clutch size. The distance between the points represents the total range of egg sizes expected for that clutch size.

lationship between offspring size and fitness. This means that they can be tested quantitatively with relative ease in any organism that produces small (but variable) clutch sizes. Consequently, cases where the invariant rules do not hold quantitatively can suggest that the fundamental assumptions of clutch size models do not hold. However, despite this possible wide applicability, there has yet to be a comprehensive, experimental test of these invariant predictions (although some tests have been done; see Charnov et al. 1995; Downhower and Charnov 1998; Mayhew 1998; West et al. 2001).

Our aim here is to carry out an explicit experimental test of the predictions and assumptions of the invariant rules. We use a small, freshwater crustacean, *Daphnia magna*. This is an excellent model organism for testing the invariant rules because clutch sizes can be in the relevant range (1–10); we can experimentally manipulate, with extreme precision, the amount of resources a female has for reproduction by varying the amount of available food; and they are clonal, which allows us to compare genetically identical individuals in the absence of paternal effects on offspring.

We use egg volume as our measure of resource allocation on the basis that egg size correlates with eventual neonate size (Goulden et al. 1987; Ebert 1993; Lampert 1993) and that egg size/offspring size correlates with fitness (Tessier and Consolatti 1989; Gliwicz and Guisande 1992), suggesting that larger eggs have more resources. We set out to maximize variation in egg size within each clutch size by maximizing the variation in resources (food) within each clutch size. We accomplish this by having many food treatments and assigning only one *Daphnia* per treatment. Our statistical tests compare egg volumes between clutches of different sizes, not between food treatments. Because *Daphnia* clutch size is correlated with food availability, we chose food treatments that would result in first clutches of between one and 10 offspring on the basis of the results of a pilot study.

Our specific aims were to test whether variation in egg size decreases with increasing clutch size (Ebert 1994; Charnov and Downhower 1995; Charnov et al. 1995), whether the range of egg sizes follows Charnov et al.'s invariant rule (1995; Charnov and Downhower 1995; eq. [1]), whether clutch size scales linearly with total resources devoted to reproduction as predicted by the Downhower and Charnov invariant rule (1998), and whether resources are allocated equally within a clutch, which is an assumption implicit in all small clutch size models.

Material and Methods

Study Organism

We used a clone of *Daphnia magna* that had originated in the Gaarzerfeld pond, northern Germany. *Daphnia magna* from this pond have been the focus of numerous studies of parasitism and life-history evolution (e.g., Little and Ebert 2001; Little et al. 2002). Throughout, *Daphnia* were fed on chemostat-grown cultures of the green algae *Scenedesmus* sp. and kept in incubators (20°C, 14L : 10D). We raised three generations of *Daphnia* in jars containing 200 mL *Daphnia* media, five *Daphnia* per jar, with 1.5×10^7 algal cells per day per jar (for 84 jars × 5 *Daphnia*/jar = 420 *Daphnia*) to equilibrate the conditions of the *Daphnia* and control for maternal and grandmaternal effects. For each new generation, we combined all neonates produced over a 24-h period (first-clutch neonates were not used) and randomly allocated neonates to jars. We changed *Daphnia* media every other day. *Daphnia* media was a modified version of the Aachener Daphnien Medium described by Klüttgen et al. (1994).

Experiment

We collected all offspring produced over a 15-h period from the third generation (first-clutch neonates were not used), placed them in separate jars containing 200 mL *Daphnia* media, and randomly assigned them to a food level. Food treatments ranged between 1.00×10^4 and 3.50×10^6 algal cells per day. Each treatment differed from the nearest food treatments by 1.00×10^4 algal cells, re-

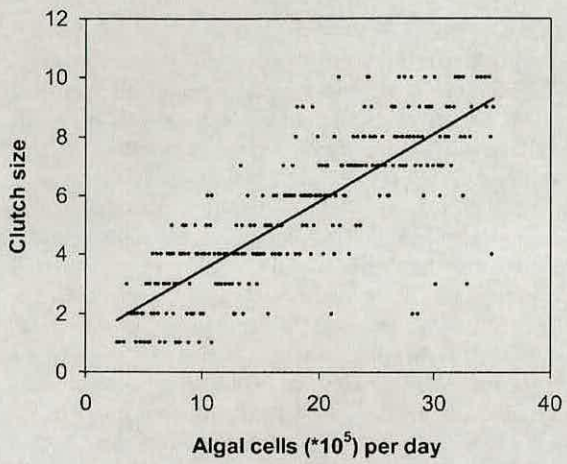

Figure 2: Relationship between food treatment and clutch size. Linear trend line included ($y = 0.2337x + 1.1147$, $r^2 = 0.663$).

sulting in 350 food treatments with one *Daphnia* per food treatment. The *Daphnia* jars were in trays, 12 jars to a tray. We randomized the location of the *Daphnia* within trays and the location of the trays within the incubator. We systematically rotated the jars within the trays and the trays within the incubator each day. We changed the *Daphnia* media in all the jars every other day. We checked the *Daphnia* twice per day, morning and evening, and recorded deaths. When the first clutch of eggs was produced, the mothers (still containing eggs) were photographed using a camera attached to a dissecting microscope. The eggs were then counted, removed, and photographed. All eggs were in stage 1 of development as described by Threlkeld (1979). Later, the photographs were used to measure mother length (top of head to base of spine) and egg diameter. Because many of the eggs were oval in shape, both length (longest axis) and width (perpendicular across middle of length) of the eggs were measured. Egg volume was estimated by modeling the eggs as an ellipsoid shape as described by equation (2):

$$\text{volume} = \frac{4}{3} \times \text{length} \times \text{width}^2. \qquad (2)$$

Data were collected for only the first clutch of eggs.

Statistics

We used the SAS system, release 8.0 (SAS Institute 1999), for all analyses not requiring resampling. Resampling was done in Microsoft Excel 2000 using the Poptools add-in (Hood 2002). When appropriate, we averaged egg volume over clutch to avoid pseudoreplication. We used generalized linear regression to test the relationship between clutch size and food level and between egg size and food level. Because variance was heterogeneous, we used a Spearman's rank correlation to test whether egg volume (averaged over clutch) changed with clutch size.

We calculated the variance in egg volume for each clutch size (using egg volumes averaged over clutch) and used an ordered heterogeneity (OH) test to evaluate whether variance decreased with increasing clutch size. The OH test combines the P value from the Bartlett homogeneity of variance test (P_{Bartlett}) with the Spearman's rank correlation coefficient (r_s) to calculate the OH statistic (r_sPc) as follows: $r_sPc = r_s \times (1 - P_{\text{Bartlett}})$ (Rice and Gaines 1994).

We used data on the mean egg volume (averaged over clutch) to test whether range in egg volume decreased as predicted by Charnov et al.'s invariant rule (1995; Charnov and Downhower 1995). We tested equation (1) for $\{i, j\} = \{2, 1\}$ through $\{10, 9\}$. We estimated the value of the invariant and the 95% confidence intervals for the left-hand side of equation (1) by bootstrapping 10,000 resamples of egg volume for each clutch size, as described by West et al. (2001). Because sample size affects range, we used the smaller sample size of the two clutch sizes, i and j, for both the numerator and denominator for each test.

Downhower and Charnov's invariant rule (1998) predicts that going from a clutch size of C to a clutch size of $C + 1$ requires an invariant increase in resources allocated to reproduction irrespective of C. This would lead to a linear relationship between clutch size and resources devoted to reproduction. We tested this prediction by using

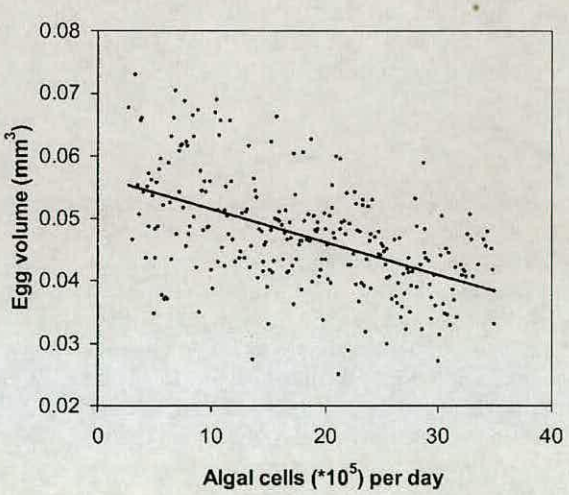

Figure 3: Relationship between food treatment and egg volume. Linear trend line included ($y = -0.0005x + 0.0567$, $r^2 = 0.260$).

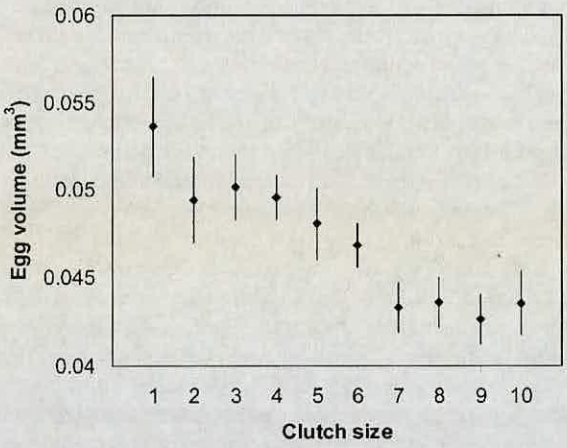

Figure 4: Mean egg volume for clutches of size one to 10. Bars are ±SEM.

generalized linear regression to relate clutch size to resources devoted to reproduction (number of eggs in a clutch multiplied by the mean egg volume for that clutch). We tested for nonlinearity by determining the significance of adding a quadratic (squared) term to the model.

To test whether resources are allocated equally within clutches, we followed the randomization procedure of West et al. (2001). Specifically, we calculated the mean within-clutch variance for each clutch size. Then, within each clutch size, we randomly allocated eggs to clutches and calculated the mean within-clutch variance of our randomized clutches. We repeated this procedure 1,000 times and tested whether the observed variance differed from the variance of our randomized clutches. This procedure was done separately for each clutch size.

Results

No reproduction was seen in animals receiving fewer than 2.70×10^5 algal cells per day, nor was it seen in approximately 8% of *Daphnia* receiving more than this amount of food. We included only *Daphnia* that produced clutches of ≤10 eggs in analyses because few *Daphnia* (<5%) produced >10 eggs. Our analyses contain data from 274 *Daphnia* and 1,336 eggs. We observed the *Daphnia* for 21 d after being placed in their treatments, during which time all *Daphnia* had either produced a clutch of eggs or died (96% of those *Daphnia* that produced eggs did so within the first 14 d of the experiment). Clutch size increased with increasing food ($F = 534.18$, df = 1, 272, $P < .0001$; fig. 2). Egg volume decreased with increasing food ($F = 89.2$, df = 1, 259, $P < .0001$; fig. 3) and decreased with increasing clutch size ($r_s = -0.34$, $P < .0001$, $n = 261$; fig. 4).

As predicted (Ebert 1994; Charnov and Downhower 1995; Charnov et al. 1995; fig. 1), variation in mean egg size (between clutches) did indeed decrease with increasing clutch size (OH test: Spearman r_s value = −0.87, Bartlett P value = 0.11, $r_sPc = -0.77$, two-tailed P value < .001; fig. 5). We used a random resampling technique to test the extent to which this led to a decrease in range of mean egg size with clutch size, as predicted by Charnov et al.'s invariant rule (1995; Charnov and Downhower 1995). The 95% confidence interval of the decrease in range included the invariant value predicted by equation (1) for all clutch sizes tested (table 1). However, confidence intervals were large, and in all cases, the 95% confidence intervals included 1. Consequently, when considering each pair of clutch sizes (i.e., comparing C with $C + 1$), individually there was no statistically significant change in range. Overall, six of the nine observed values were larger than the expected values. This lack of a close fit to Charnov et al.'s prediction is also shown by the fact that the range did not show a symmetrical convergence around the mean; while the ninetieth percentile of mean egg size significantly decreased with increasing clutch size (slope = −0.0019 mm³/clutch size, $r_s = -0.92$, $n = 10$, $P = .0002$), the tenth percentile did not significantly increase with increasing clutch size (slope = 0.0003 mm³/clutch size, $n = 10$, $r_s = -0.61$, $P = .060$; fig. 5).

We carried out two further tests to ensure the robustness of the negative relationship between variance in egg vol-

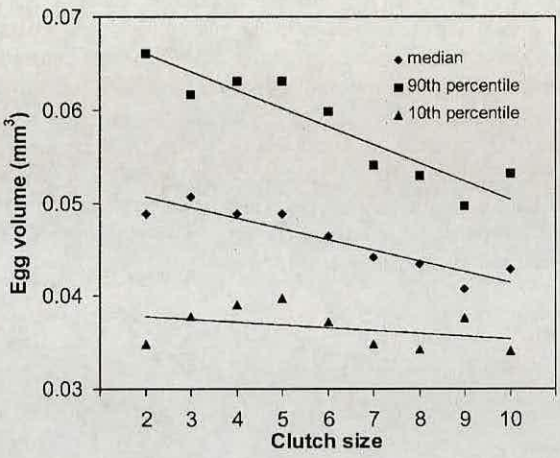

Figure 5: Median (*diamonds*), ninetieth percentile (*squares*), and tenth percentile (*triangles*) for egg volume for clutches of size one to 10. Linear trend lines included (median: $y = -0.0011x + 0.053$, $r^2 = 0.851$; ninetieth percentile: $y = -0.0019x + 0.0699$, $r^2 = 0.853$; tenth percentile: $y = -0.0003x + 0.0384$, $r^2 = 0.148$).

Table 1: Test of equation (1)

Clutch sizes compared	Sample size	Expected $\frac{C_i}{C_j}$	Observed $\frac{I_{\max i} - I_{\min i}}{I_{\max j} - I_{\min j}}$	Lower confidence limit (2.5%)	Upper confidence limit (97.5%)
2 vs. 1	15	.5	1.09	.71	1.65
3 vs. 2	22	.67	.83	.57	1.10
4 vs. 3	23	.75	.99	.70	1.41
5 vs. 4	24	.80	1.28	.72	1.78
6 vs. 5	24	.83	.72	.46	1.21
7 vs. 6	35	.86	1.41	.72	1.85
8 vs. 7	28	.88	.70	.43	1.39
9 vs. 8	17	.89	.81	.37	1.40
10 vs. 9	16	.90	1.21	.64	2.68

Note: The observed values (median of the 10,000 resamples) and 95% confidence intervals for $(I_{\max i} - I_{\min i})/(I_{\max j} - I_{\min j})$ were obtained by resampling the egg volume data (averaged over clutch).

ume and clutch size. First, we tested that this pattern was not simply a statistical artifact due to averaging over more eggs as clutch size increased. To do this, we randomly chose one egg from each clutch and calculated the regression line relating variance in egg volume to clutch size. We repeated this 100 times. All 100 regression lines had a negative slope, leading us to conclude that variance does indeed decrease with increasing clutch size ($P < .01$). Second, we tested that the relationship did not arise merely as a result of differences in egg shape. We did this by testing for relationships between clutch size and egg length and between the variance in egg length and clutch size. The results of both these tests were similar to those reported for egg volume and supported the robustness of our results.

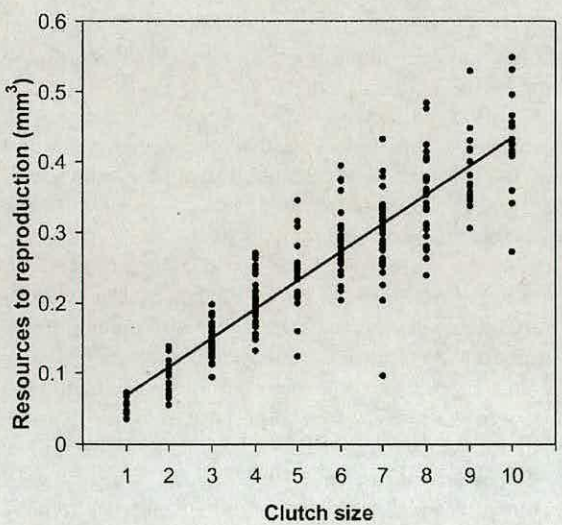

Figure 6: Relationship between clutch size and resources devoted to reproduction (number of eggs in a clutch multiplied by the mean egg volume for that clutch). Linear trend line included ($y = 0.0406x + 0.0275$, $r^2 = 0.826$).

Downhower and Charnov's invariant rule (1998) predicts that the amount of extra resources needed to produce an additional egg remains constant over clutch size. This therefore predicts a linear relationship between resources devoted to reproduction and clutch size. Resources devoted to reproduction were significantly related to clutch size (fig. 6; $F = 35.1$, df $= 1, 259$, $P < .0001$), with a linear regression explaining 82.6% of the variation in the data. We then tested for nonlinearity by adding a squared term (clutch size squared) to the model; the squared term did not significantly improved the model ($F = 3.12$, df $= 1, 258$, $P = .078$).

Clutch size models assume that each egg in a clutch receives equal resources. Our randomization procedure allowed us to test this statistically by comparing variation between eggs from the same clutch to that of eggs from different clutches (within the same clutch size). Eggs from the same clutch were more similar in volume than were eggs from different clutches ($P < .01$) for every clutch size tested (two to 10), supporting the hypothesis that mothers allocate resources relatively equally between eggs in a clutch (table 2).

Discussion

We have carried out an experiment designed explicitly to test the predictions of small clutch size models and in particular their invariant rules (Ebert 1994; Charnov and Downhower 1995; Charnov et al. 1995; Downhower and Charnov 1998; fig. 1). In support of these invariants, we found that variance in egg volume decreased with clutch size (fig. 5) and clutch size showed a linear relationship to resources devoted to reproduction (fig. 6). As assumed

Table 2: Observed mean within-clutch variance in egg volume compared to sampled mean within-clutch variance in egg volume by clutch size

Clutch size	Sample size	Observed mean variance ($\times 10^{-5}$)	Sampled mean variance ($\times 10^{-5}$)	Lower confidence limit (2.5%) ($\times 10^{-5}$)	Upper confidence limit (97.5%) ($\times 10^{-5}$)	*P* value
2	22	2.81	11.25	6.46	16.18	<.01
3	23	2.68	9.51	6.76	12.62	<.01
4	45	2.65	7.87	6.58	9.34	<.01
5	24	4.93	13.17	10.71	15.95	<.01
6	35	4.55	8.27	6.15	10.28	<.01
7	36	4.48	8.77	7.58	10.44	<.01
8	28	4.61	8.82	7.66	10.40	<.01
9	17	3.52	6.60	5.65	7.77	<.01
10	16	3.47	8.19	7.41	9.06	<.01

Note: The 95% confidence intervals are for sampled values.

by these models, we found that eggs from the same clutch were more similar in volume than were eggs from different clutches (within a clutch size), suggesting that mothers allocate resources relatively equally between eggs in a clutch (table 2). However, mean egg volume decreased with clutch size (fig. 4), and the rate at which the range of egg volumes decreased with clutch size did not show a strong fit to the predictions of the Charnov et al. invariant rule (1995; Charnov and Downhower 1995; fig. 1) regarding how the range of egg volumes should change with clutch size (table 1). Specifically, although the 95% confidence intervals of our data include the values predicted by equation (1), the confidence intervals are large and in all cases include 1 (table 1). Moreover, the tenth and ninetieth percentiles of mean egg size per clutch did not converge symmetrically around the mean with increasing clutch size (fig. 5). There are several possible hypotheses that could explain why range of egg sizes decreased more slowly than expected.

First, there might exist a lower boundary on egg volume below which eggs are not viable. A lower limit on egg size in *Daphnia* has been postulated previously (Glazier 1992), and at small egg sizes, there is a strong positive correlation between egg size and likelihood of hatching in *Daphnia pulex* (Bell 1983). The consequences of a minimum egg size is that in some circumstances, *Daphnia* females would reduce their clutch size by one rather than produce eggs smaller than this minimum egg volume, hence decreasing the possible egg volume/egg number combinations. Consistent with this, we found that while the ninetieth percentile of egg volumes decreased with increasing clutch size, there was no change in the tenth percentile (fig. 5). A similar pattern has been observed in a fish (Charnov et al. 1995).

Second, the optimal egg size may vary with food and resource availability and therefore clutch size. In our study, mean egg volume decreased with increasing clutch size. Decreasing egg (or neonate) size with increasing clutch size (or increasing food availability because clutch size and food availability are correlated) has been found previously in *Daphnia* (e.g., Bradley et al. 1991; Gliwicz and Guisande 1992; Ebert 1993; although other patterns have also been observed, e.g., Boersma 1995; Lynch 1989; Tessier and Consolatti 1991; Glazier 1992). Equation (1) assumes that mean egg size remains constant. Our results are consistent with the hypothesis that mothers in bad environments (in this case, low food) produce larger eggs than do mothers in good environments (high food). This would occur if the fitness benefit of being a large offspring is greater at lower food (Hutchinson 1951; Green 1966; Goulden et al. 1987; Gliwicz and Guisande 1992; Ebert 1994).

Third, the relationship between resources allocated to an egg and egg volume might be nonlinear. Egg volume is correlated with offspring fitness (as discussed in the introduction to this article), suggesting that larger eggs do indeed receive more resources than do smaller eggs. However, the carbon : nitrogen ratios in neonates differ with the mothers' food availability in *Daphnia pulicaria* (Tessier and Consolatti 1991), suggesting that resource partitioning may differ between eggs of different sizes. In addition, size can affect predation risk for *Daphnia*, and *Daphnia* can adjust age and size at maturity in line with predation risk (Weider and Pijanowska 1993; Stibor and Lüning 1994). In other organisms, complicated size-fitness relationships have been described, for example, where fitness depends on an individual's size relative to competitors (Westoby et al. 1992; Rees and Westoby 1997).

Fourth, a female might be simultaneously optimizing the amount of resources that she allocates to reproduction with the resources allocated elsewhere, such as to growth. If this is the case, then she might not be constrained to put a fixed amount into reproduction, dependent on food levels.

Conclusions

Quantitative life-history predictions often require a detailed knowledge of life-history details that are difficult to measure. Recently, it has been shown that this problem can in some cases be resolved through the use of a dimensionless approach (Charnov 1993, 1997). This method can predict life-history invariants that permit quantitative tests in the absence of a detailed knowledge of underlying trade-offs and without the inclusion of biological complexities. As well as the small clutch size invariants tested here, life-history invariants have been successfully utilized to explore topics as varied as age at maturity in parasitic nematodes (Gemmill et al. 1999), timing of sex change in fish (Charnov and Skuladottir 2000; Allsop and West 2003*a*, 2003*b*), and life-history characteristics (age at maturity, cellular maintenance rate) in mammals (Charnov 2001).

In this study, Charnov et al.'s invariants (1995; Charnov and Downhower 1995; Downhower and Charnov 1998) have proven useful for expanding our knowledge specifically about *Daphnia* reproduction and generally about resource investment into offspring. We found qualitative support for the prediction that variance in egg volume should decrease with increasing clutch size (Ebert 1994; Charnov and Downhower 1995; Charnov et al. 1995; fig. 5). Where the data do not fit the invariant predictions quantitatively has led to several hypotheses about *Daphnia* reproduction and about resource allocation in general. These observations require further investigation. In particular, Is there a minimum viable egg volume? Does the relationship between egg volume and offspring fitness vary with food availability and thus clutch size? To what extent are our findings generalizable to more species? In addition, it would also be extremely useful to explore the theoretical implications of a minimum viable egg volume and variation in the optimal egg volume with clutch size.

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Host immune status affects maturation time in two nematode species – but not as predicted by a simple life-history model

M. A. GUINNEE[1]*, A. W. GEMMILL[1]†, B. H. K. CHAN[1], M. E. VINEY[2] *and* A. F. READ[1]

[1] *Institute of Cell, Animal and Population Biology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, UK*
[2] *School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, UK*

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SUMMARY

In theory, the age at which maturation occurs in parasitic nematodes is inversely related to pre-maturational mortality rate, and cross-species data on mammalian nematodes are consistent with this prediction. Immunity is a major source of parasite mortality and parasites stand to gain sizeable fitness benefits through short-term adjustments of maturation time in response to variation in immune-mediated mortality. The effects of thymus-dependent immune responses on maturation in the nematode parasites *Strongyloides ratti* and *Nippostrongylus brasiliensis* were investigated using congenitally thymus-deficient (nude) rats. As compared with worms in normal rats, reproductive maturity of parasites (presence of eggs *in utero*) in nude rats occurred later in *S. ratti* but earlier in *N. brasiliensis*. Immune-mediated differences in maturation time were not associated with differences in worm length. Thymus-dependent immunity had no effect on pre-maturational mortality. Results are discussed in relation to theoretical expectations and possible explanations for the observed patterns in parasite maturation.

Key words: maturation time, *Nippostrongylus brasiliensis*, nude rats, parasitic nematodes, *Strongyloides ratti*.

INTRODUCTION

For most animals, life begins with a period of pre-maturational somatic growth, the duration of which can have a large impact on fitness. For example, generation time, mortality, body length and fecundity are themselves often functions of maturation time. The age of reproductive maturity is therefore of central biological importance and likely to be a major target of natural selection (Roff, 1992; Stearns, 1992).

For parasitic nematodes, an optimality model of maturation time has been developed based on the relationship of body size and fecundity (Gemmill, Skorping & Read, 1999). According to this model, extended growth before reproduction results in larger size which enhances fecundity, but also entails a heightened risk of death prior to reproduction. Thus, natural selection favours smaller, more rapidly maturing worms when the resulting increase in survival outweighs size-related reductions in fecundity. Conversely, when pre-maturational mortality is sufficiently low, the fecundity benefits of large size tip the balance in favour of delayed maturity. This model explains about half the variation in maturation time across a wide range of mammalian gastrointestinal nematodes (Gemmill *et al.* 1999). It might also explain variation in maturation time within species of nematodes (Gemmill *et al.* 1999).

In some free-living species, individuals adjust maturation time adaptively in response to environmental variation. For example, *Daphnia* (small freshwater crustacea) adjust their age and size at maturity in the presence of a predator, and are even able to discriminate between predators that prey on *Daphnia* of different sizes (and presumably of different ages). *Daphnia* exposed to chemicals from predators that prey on small *Daphnia* mature later and larger, while chemicals from predators that prey on large *Daphnia* result in earlier maturation at a smaller size (Weider & Pijanowska, 1993; Stibor & Luning, 1994). This is a phenotypic response and the magnitude of the response can differ between *Daphnia* clones (Weider & Pijanowska, 1993). Theoretical studies show that such a capacity can evolve where environmental variability has large effects on fitness and where individuals have reliable cues that indicate the nature of that variability (e.g. Moran, 1992; Scheiner, 1993).

Within a host population, immunocompetence varies with factors such as nutritional status, age, pregnancy and stress (e.g. Anderson, 1988; Stear & Wakelin, 1998). Consequently, the life-expectancy of a developing nematode is likely to depend very much on the type of host in which it finds itself. A worm that could assess the likely efficacy of the immune system of a host, and vary its life-history accordingly, could maximize its life-time reproduction

* Corresponding author: Institute of Cell, Animal and Population Biology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, UK. Fax: +44 (0)131 650 6564. Tel: +44 (0)131 650 6468. E-mail: meghan.gannon@ed.ac.uk

† Current address: Department of Clinical and Health Psychology, Austin and Repatriation Medical Centre, Repatriation Campus, Banksia Street, Heidelberg West, Victoria 3081, Australia.

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whatever the immune competence of the host. In principle, components of the host immune system could provide the necessary cues. There is evidence that components of the host immune system might play a role in controlling development in schistosomes (Amiri *et al.* 1992; Davies *et al.* 2001; Ravindran, 2001). The nematode *Caenorhabditis elegans* can enter a non-feeding, stress-resistant stage, called a dauer larva, which can survive long past the typical *C. elegans* life-span (Golden & Riddle, 1982; Riddle & Albert, 1997, and references therein). Many nematodes of veterinary importance (e.g. *Ostertagia* spp., *Haemonchus contortus*, *Trichostrongylus* spp.), can likewise enter a diapause state, in response to cool temperature or other environmental factors (Anderson, 1992, and references therein).

The thymus-dependent immune system can substantially decrease *Strongyloides ratti* and *Nippostrongylus brasiliensis* reproduction and/or survival (Dawkins, Mitchell & Grove, 1982; Kassai, 1982, and references therein; Smith, Ovington & Bryant, 1991; McKay *et al.* 1995; Gemmill, Viney & Read, 1997). Consequently there should exist strong pressure to optimize life-history parameters in line with parasite mortality resulting from the host immune response. Can nematodes respond to the presence of the host immune system as *Daphnia* respond to the presence of predators?

Here we investigate whether and how nematode maturation time is dependent on host immune status in *S. ratti* and *N. brasiliensis*, two intestinal nematode parasites of rats. We make use of congenitally athymic (nude) rats, which are incapable of mounting thymus-dependent immune responses. We quantify variation in parasite establishment, maturation time and body length to explore the following questions. Do nematode parasites delay maturation in immunodeficient hosts? If so, is there a size benefit associated with the delay?

MATERIALS AND METHODS

Parasites

S. ratti and *N. brasiliensis* are gastrointestinal nematodes that are natural parasites of rats. Adult parasites of both species inhabit the mucosa of the small intestine. In the intestine, *S. ratti* are exclusively parthenogenetic females (Viney, 1994), while *N. brasiliensis* reproduce sexually (Anderson, 1992). Eggs of both species are shed into the intestine and pass with host faeces to the external environment where further development takes place resulting in infective 3rd-stage larvae (iL3s). When an iL3 comes into contact with a rat, it burrows through host skin and migrates to the intestine. The majority of worms have arrived at the gut by 50 h (*N. brasiliensis*) or 70 h (*S. ratti*) post-skin penetration (Tindall & Wilson, 1990).

Infections

For both *S. ratti* and *N. brasiliensis* experiments, 100 iL3s were counted under a dissecting microscope and administered subcutaneously in saline (0·80% w/v NaCl). The syringe and needle were then flushed with saline, and the number of remaining worms counted. In this way, a precise inoculum size could be calculated.

Strongyloides ratti

The isofemale line ED5 (Viney, 1996) was used and was maintained by serial passage in Wistar rats (B & K Universal, UK). Experimental animals were female rats, aged 6–10 weeks (Harlan, UK). Fifteen nude (Hsd:*rnu/rnu*) and 15 heterozygous (Hsd:*rnu/+*) rats were infected with 100 iL3s as described above. We henceforth refer to the heterozygous rats as 'normal' rats, as they have fully functioning immune systems. To sample adult worms in the gut, 5 nude and 5 normal rats were euthanized on each of days 4, 5 and 6 post-infection (p.i.). The experiment was carried out in 2 experimental blocks, the first block consisting of 2 normal and 2 nude rats euthanized on each of days 4, 5, and 6 p.i., and the second block consisting of 3 normal and 3 nude rats euthanized on each of days 4, 5, and 6 p.i.

Nippostrongylus brasiliensis

Parasites were obtained from R. M. Maizels, University of Edinburgh, and maintained in female nude rats (HsdHan:*rnu/rnu*; Harlan, UK). Experimental animals were female rats, aged 9 weeks (Harlan, UK). Fifteen nude (HsdHan:*rnu/rnu*) and 15 normal (HsdHan:*rnu/+*) rats were infected with 100 iL3s as described above. To sample adult worms in the gut, 5 nude and 5 normal rats were euthanized on each of days 5, 6 and 7 p.i. This experiment was not done in blocks.

Reproductive output

Strongyloides ratti. To measure parasite reproductive output, faeces from the animals euthanized on day 6 p.i. were collected overnight on days 3 and 4 p.i. Faecal cultures were prepared as described by Viney, Mathews & Walliker (1992). After 2 days incubation at 25 °C free-living stages were washed from culture plates, collected and counted under a binocular microscope as described by Gemmill *et al.* (1997).

Nippostrongylus brasiliensis. To measure parasite reproductive output, faeces were collected from every rat overnight on days 5, 6 and 7 p.i. Egg output per night was estimated using a modified McMaster's technique.

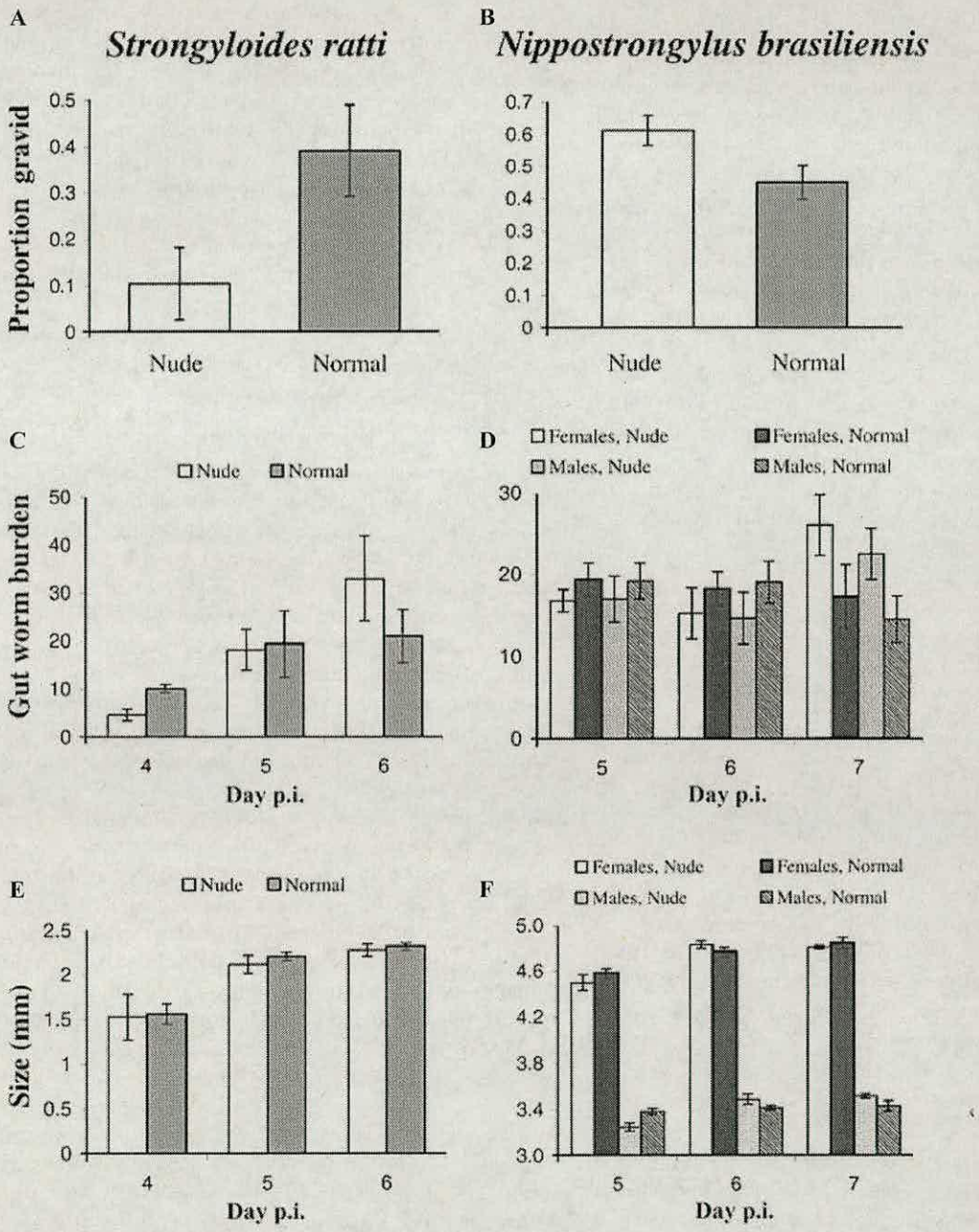

Fig. 1.Parasites from nude rats and normal rats. Graphs A and B show the proportion of worms with eggs *in utero* day 4 (graph A, *Strongyloides ratti*) or day 5 (graph B, *Nippostrongylus brasiliensis*) for nude and normal rats (averaged over rat). Graphs C and D show the number of worms per rat on days 4, 5 and 6 p.i. (graph C, *S. ratti*) or 5, 6 and 7 p.i. (graph D, *N. brasiliensis*) for nude and normal rats. Graphs E and F show the mean body length (mm) of adult worms on day 4, 5 and 6 p.i. (graph E, *S. ratti*) or 5, 6 and 7 p.i. (graph F, *N. brasiliensis*). Each bar in graphs A–F is the mean of $n=5$ rats, except for *S. ratti*, nude, day 6 p.i. which is the mean of 4 rats. Errors are ± 1 S.E.M.

worms were broken or too twisted to measure accurately, approximately 10% could not be measured.

Reproduction. Eggs were first detected in the faeces of all rats on day 6 p.i. Nude and normal rats had similar egg outputs on day 6 p.i. ($F_{1,18}=0{\cdot}00$, $P=0{\cdot}97$); but nude rats expelled more eggs on day 7 p.i. ($F_{1,7}=13{\cdot}82$, $P=0{\cdot}0075$). On day 5 p.i., a higher proportion of female worms were gravid in nude rats than normal rats (Fig. 1B; host immune status: $Q_W=5{\cdot}51$, D.F. $=1$, $P=0{\cdot}019$). By day 6 p.i., nearly all female worms (96%) had eggs *in utero*.

Adult worm recovery. Of all the *N. brasiliensis* recovered in this study, 85·6% were in the anterior, 13·3% were in the middle and 1·1% were in the posterior third of the small intestine. Fifty-two percent of recovered worms were female. Sex ratios did

Adult worms

Strongyloides ratti. Intestinal parasite numbers (worm burdens) were determined as described by Gemmill, Viney & Read (2000). Briefly, the method is as follows. Immediately after rat death, the small intestine was excised, opened longitudinally and rinsed briefly in tap water. Each small intestine was then divided into 3 approximately equal parts and each part incubated separately in saline (0·8% w/v NaCl solution). After 2 h incubation at 37 °C, each portion of small intestine was vigorously rinsed and backwashed with fresh saline in order to detach any remaining parasites. Recovered parasites were killed and straightened by immersion in boiling formalin solution (10% formal saline). Parasites were then transferred to a droplet of glycerol on a microscope slide, encircled with fine ground glass, covered with a cover-slip and sealed with a polyurethane-based sealant.

Nippostrongylus brasiliensis. Intestinal parasites were recovered as described by Kassai (1982). Briefly, the method is as follows. The small intestine of each rat was removed immediately after death and cut into 3 approximately equal sections. Each section was then cut into 3 pieces, slit along its length and placed in a gauze sack. The sack was suspended in saline in a test-tube and incubated at 37 °C for 2 h. Worms migrated through the gauze and collected at the bottom of the tube. Recovered parasites were killed and fixed by immersion in boiling formalin solution (7% formalin). They were transferred to anhydrous glycerine according to Seinhorst's (1959) method as modified by De Grisse (1969). The worms were then placed in a drop of glycerine on a slide and sealed with wax.

Parasite measurements

Parasites of both species were examined under a binocular microscope. Sex (for *N. brasiliensis*) and presence or absence of eggs *in utero* were recorded for each worm. The body length of each worm was measured using the PC_IMAGE software package (version 2.2.01: Foster Finlay Associates, UK) and a JVC video-camera module (model TK 1270).

Statistical analyses

Worm measurements were averaged over rat to avoid pseudoreplication. The SAS System: Release 8.0 (SAS Institute) was used for all statistical analyses. Data were analysed using analyses of variance (ANOVAs), except for proportion data (worm sex ratio and the proportion of female worms with eggs *in utero*) which were analysed using the Wald chi-squared statistic (Q_W) from the proc logistic command in the SAS system. Host immune status, day p.i., initial innoculum size, gut worm burden and experimental block were included in maximal models where appropriate. Significant effects involving block (replicate) are thus controlled for, but they are of little intrinsic interest in their own right, so that they are reported only where they qualitatively affect the conclusion. Where possible, all 2- and 3-way interactions were also included in maximal models. Non-significant terms ($P>0·05$) were eliminated from models using backwards elimination (Crawley, 1993).

RESULTS

Strongyloides ratti

Inoculum sizes ranged from 88 to 100 larvae with a mean of 94·8 and did not differ with host immune status, day p.i. or the interaction between the two ($P>0·05$). One of the nude rats scheduled to be euthanized on day 6 p.i. died of unknown causes prior to being euthanized; this rat was excluded from analyses. A total of 499 worms were included in this study. An attempt was made to measure every worm from every rat; however, because some of the worms were broken or too twisted to measure accurately, approximately 45% could not be measured.

Reproduction. Larvae were first detected in the faeces of all rats on day 4 p.i. Worm output on day 4 p.i. was lower in nude rats as compared to normal rats, but this difference was non-significant ($F_{1,7}=3·57$, $P=0·10$). The proportion of egg-bearing worms in nude rats was significantly less than that in normal rats, indicating a delay in the onset of reproductive maturity in nude rats (Fig. 1A; $Q_W=5·28$, D.F. $=1$, $P=0·022$). Nearly all worms (>99%) recovered on days 5 and 6 p.i. had eggs *in utero*.

Adult worm recovery. Worm burdens increased with day p.i. faster in nude rats than in normal rats (Fig. 1C; day p.i.: $F_{2,17}=6·04$, $P=0·010$; host immune status: $F_{1,17}=0·38$, $P=0·54$; host immune status by day p.i. interaction: $F_{2,17}=4·43$, $P=0·028$).

Average worm length increased with day p.i. (Fig. 1E; $F_{2,24}=31·92$, $P<0·0001$). Parasite body length was not significantly affected by host immune status (Fig. 1E; host immune status: $F_{1,23}=0·50$, $P=0·49$; host immune status by day p.i. interaction: $F_{2,21}=0·03$, $P=0·97$).

Nippostrongylus brasiliensis

Inoculum sizes ranged from 80 to 107 larvae with a mean of 97·23 and did not differ with host immune status, day p.i. or the interaction between the two ($P>0·05$). A total of 1099 worms were included in this study. An attempt was made to measure every worm from every rat; however, because some of the

not vary significantly with day p.i., host immune status or the interaction between the two ($P>0{\cdot}05$). There was a slight decrease in gut worm burdens (total number of male and female worms) in normal rats as the infection progressed; this trend was not seen in nude rats (Fig. 1D; host immune status by day p.i. interaction: $F_{2,23}=5{\cdot}93$, $P=0{\cdot}0084$).

Female worm length increased with day p.i., but was not affected by host immune status (Fig. 1F; day p.i.: $F_{2,26}=26{\cdot}54$, $P<0{\cdot}0001$; host immune status: $F_{1,25}=0{\cdot}05$, $P=0{\cdot}83$; host immune status by day p.i. interaction $F_{2,23}=1{\cdot}74$, $P=0{\cdot}20$). Male worm length increased with day p.i., with those in nude rats increasing in length faster than those in normal rats (Fig. 1F; day p.i.: $F_{2,24}=12{\cdot}60$, $P=0{\cdot}0002$; host immune status: $F_{1,24}=0{\cdot}10$, $P=0{\cdot}75$; host immune status by day p.i. interaction $F_{2,24}=6{\cdot}62$, $P=0{\cdot}0051$).

DISCUSSION

Gemmill *et al.* (1999) predicted that individual parasitic nematodes stand to gain sizeable fitness benefits by adjusting maturation time in line with prevailing levels of immune-imposed, pre-maturational mortality. Where such mortality is high, more rapid maturation can enhance fitness by maximizing survival to reproductive age. Where it is low, extended growth will pay off as increased size resulting in increased egg production.

In this study, exposure to the thymus-dependent immune system did indeed affect *S. ratti* and *N. brasiliensis* maturation time, but the two species experienced opposite responses. *S. ratti* matured later in nude rats than in normal rats, as found previously (Dawkins *et al.* 1982; Gemmill *et al.* 1997, 2000). Conversely, *N. brasiliensis* matured later in normal rats. The alterations in the timing of reproduction did not have any subsequent effect on worm length in either species. Thus, our study does not support our hypothesis that parasites experiencing less immune-dependent mortality mature later to increase size and optimize reproduction: *N. brasiliensis* actually matured earlier and the delay in maturation of *S. ratti* did not result in larger worms.

The model assumes that negligible worm growth occurs after maturation. We used the length of iL3s and the average female worm length on day 4 p.i. (*S. ratti*) and day 5 p.i. (*N. brasiliensis*) to estimate the average increase in length per worm per day before maturation (maturation was defined as occurring on day 5 p.i. for *S. ratti*, on day 6 p.i. for *N. brasiliensis*). We then calculated the average increase in length per worm per day post-maturation (growth between days 5 and 6 p.i. for *S. ratti*, between days 6 and 7 p.i. for *N. brasiliensis*). We found that the pre-maturational growth (mm/day) was at least 3 (*S. ratti*) to 10 (*N. brasiliensis*) times greater than the post-maturational growth, supporting our assumption.

There are many possible reasons why this study did not support our life-history hypothesis, one of which is that the hypothesis is wrong. Alternatively, it might be that the worms are unable to appropriately sense the experimental immune environment we provided or appropriately adjust their life-history in it. Nude rats are presumably extremely rare in nature, indeed, if they occur at all, and it might be that the worms were confused by some novelty of their physiology. This could be tested by using other methods of immunosuppression.

Another possibility is that the worms are actually extremely good at predicting their life-expectancy and that our experimental manipulation does not in fact capture the situation in which alteration in maturation time would be favoured by natural selection. In these experiments, as in a previous experiment using *S. ratti* (Gemmill *et al.* 2000), there was no evidence that thymus-deficient hosts had lower pre-maturational mortality, although the increase in worms in nude rats with day p.i. might support this. In *S. ratti*, adult worm burdens on the first day of sampling were lower in nude rats, and in *N. brasiliensis* they were similar. Thus, the worms might have been correctly predicting no reduction in mortality in the nude rats. But, if so, it begs the question of why maturation times were nonetheless altered. Moreover, the larval forms of these parasites are highly susceptible to the immune response in hosts with prior exposure (Kassai, Takáts & Redl, 1974, as cited by Kassai, 1982; Gemmill *et al.* 1997). We believe that the observed lack of immune-mediated, pre-maturational parasite mortality is most likely to result from a lag between parasite invasion and host immune response in naïve hosts, rather than a lack of immune response to larval stages. Under more natural circumstances, a lack of thymus-dependent immunity should predict enhanced larval survival, and worms should delay reproduction accordingly.

Alternatively, the model might be too simple. For example, if post-maturational or size-dependent mortality are themselves important functions of maturation time, this might affect the nature of the optimal phenotypic response to shifts in the prevailing mortality schedule (Skorping & Read, 1998). If so, quantitative success of the Gemmill *et al.* (1999) model in predicting cross-species variation in age to maturity would presumably be enhanced by taking these complexities into account.

It might be significant that the highest worm counts for both parasite species occurred in nude rats on the last day of the experiment, and day p.i. was correlated with worm number for *N. brasiliensis* in nude rats (*N. brasiliensis*: $F_{2,11}=6{\cdot}12$, $P=0{\cdot}03$; *S. ratti*: $F_{1,12}=2{\cdot}31$, $P=0{\cdot}15$). It is possible that the worms in nude rats experienced slowed migration, development and reproduction in the absence of immune cues; this would not be the first such finding in

a helminth parasite (Amiri *et al.* 1992; Davies *et al.* 2001; Ravindran, 2001). If this is the case, no appreciable size benefit was associated with the delay, and therefore no evidence of an adaptive advantage was detected. The alterations in time of maturity in *S. ratti* and *N. brasiliensis* in nude rats could be a pathological consequence of life in an novel environment for which they were unprepared by evolution; alternatively, the alterations might represent adaptive short-term adjustments of the developmental schedule, the fitness consequences of which remain to be determined.

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